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# Effect of Anaerobic Root Environments on Fraxinus Pennsylvanica Marsh. And Quercus Nigra L.

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EFFECT OF ANAEROBIC ROOT ENVIRONMENTS ON FRAXINUS PENNSYLVANICA MARSH. AND QUERCUS NIGRA L.

*The Louisiana State University and Agricultural and Mechanical Col.* PH.D. 1985

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# Effect of Anaerobic Root Environments on *Fraxinus pennsylvanica* Marsh. and *Quercus nigra* L.

A DISSERTATION

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

Marine Sciences

by
Billy J. Good
B.S., Louisiana State University, 1976
M.S., Louisiana State University, 1981
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To Peggy, with all my love.

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## ABSTRACT

Responses of green ash (Fraxinus pennsylvanica Marsh.) and water oak (Quercus nigra L.) to anaerobic root environments were investigated. Initially, analytical methods pursuant to this objective were developed. An extraction solution containing DTT (dithiothreitol) and soluble polyvinylpyrrolidone proved best for oak ADH and protein recovery, while DTT and insoluble polyvinylpolypyrolidone were best for ash.

A method for the control of the soil atmosphere surrounding intact roots was developed to study the effects of elevated $CO_2$ in the anaerobic soil atmosphere of ash and oak seedlings. Ethanol, malate, ADH, ME (NADP-malic enzyme), $CO_2$, and $O_2$ within the root systems were monitored. Linear, statistically significant, increases in ADH and ME activities, and $CO_2$ and ethanol concentrations were found in ash roots. These results suggested that $CO_2$ may increase ME and ADH activities by lowering cytoplasmic pH. Increased soil $CO_2$ resulted in higher levels of ethanol and $CO_2$ in the roots of the oak than in those of the ash - an apparent expression of ash's superior flood tolerance.

A long-term (9.5 month) flooding experiment was conducted in which the root gas composition, malate, soluble protein, ADH, selected root-coating constituents and their corresponding leaf concentrations, and growth were monitored on flooded ash and oak seedlings and drained controls. The ash maintained higher oxygen and

lower $CO_2$ concentrations during flooding than the less flood-tolerant species, water oak. This was reflected in differences in root coating composition, and the superior ability of the green ash to prevent the accumulation of potentially phytotoxic compounds in the leaves.

A field study was conducted in which ash and oak seedlings were transplanted along four BLH (bottomland hardwood) transects that represented a wide range of soil-moisture, flooding regimes in Louisiana. Root coating constituents and ADH activity of green ash seedlings were assayed 1.5 years after transplanting. Oak mortality precluded analysis of this species. The deposition of root-coating materials and ADH activity were distinctly different enough from seedlings of the wet and mesic sites to be useful in the development of a two-group discriminant analysis function for site-wetness classification. The percentages of seedlings correctly grouped into the mesic and wet categories were 88.9 and 92.0, respectively.

# CHAPTER ONE

# INTRODUCTION

Wetlands, an important natural resource, are valued for their high productivity, vital role in many food chains, and importance in erosion control and recreation. Delineating the boundaries of wetlands is currently a problem. It is generally agreed that semi-permanently flooded swamps are wetlands and that wetlands probably extend into bottomland hardwood (BLH) forests. The question is, how far? Because of the relationships known to exist between waterlogged soil conditions and plant responses, vegetation can be used as a criterion in delineating the extent of wetlands. The current legal definition of wetlands illustrates this:

> "...those areas that are inundated or saturated by surface or groundwater at a frequency and duration sufficient to support,and that under normal circumstances do support a prevalence of vegetation typically adapted for life in saturated soil conditions"
> (Federal Register Vol. 42 [138] Part 323,July 19, 1977).

This definition further stimulated an already active field of scientific investigation. The question of how plants adapt to life in saturated soil conditions had been pursued by many researchers, particularly those interested in marsh plants and certain agricultural species, such as rice, grown under flooded conditions.

With the development of the idea of wetland delineation in BLH's, the adaptations of woody species to waterlogging fell under greater scrutiny. Although the changes in forest community composition attributable to differences in waterlogging duration and intensity have been extensively described, relatively little is known about the adaptive responses of individual BLH species or how these could be related to wetland delineation.

Several constraints are unique to the study of flood-adapted tree species. Since relatively little physiological work has been done on their root systems, the methods involved in enzymatic assays must be worked out de novo for practically every species. This is not a simple task because trees contain large quantities of secondary compounds, many of which are potent enzyme inhibitors. Additionally, tree-root tissue is generally much denser than that of herbaceous species which makes root-gas analysis relatively difficult. While it is known that lenticels play a role in root areation, little else is known regarding the dynamics of $CO_2$ and $O_2$ content of roots under various anaerobic conditions. Finally, the root-soil interactions of flood-tolerant BLH species under field conditions are very complex and they have simply not been addressed in the literature.

Therefore, my research had four objectives. The first was to optimize the biochemical techniques necessary to analyze anaerobic metabolism in the root tissue of green ash and water oak. The second objective was to develop a system to control soil atmospheric conditions so that root responses under controlled anaerobic

conditions could be monitored without interference from the chemical changes that accompany soil waterlogging. The third objective was to investigate root responses in controlled flooded-soil conditions. The final objective of this study was to develop a model, based on root responses, that could be related to the wetland delineation issue under a wide range of BLH field conditions.

# CHAPTER TWO

## LITERATURE REVIEW: PLANT ADAPTATIONS TO FLOODED SOIL CONDITIONS

Plant adaptations to anaerobic soil conditions have been intensively studied due to their importance in such diverse fields as plant ecology, agronomy, forestry, and the management and regulation of wetlands. Several extensive literature reviews have recently been published which summarize much of the progress in this area (e.g. see Crawford, 1978a; 1982; 1983; Hook, 1984a; 1984b; Kozlowski, 1984; Whitlow and Harris, 1979). The purpose of this review is to provide background information for the investigations reported in this dissertation.

Plant adaptations to flooding are difficult to discuss individually because it is their integrated effect that determines flood tolerance (Hook and Brown, 1973). Nevertheless, several aspects have received sufficient attention in the literature to warrant separation in the following discussion. This is for the sake of convenience only, and is not meant to imply that they are in some sense separate from each other in nature. For example, the degree of root aeration will determine the rate of aerobic respiration, and the degree to which reduced phytoxins are detoxified; and this will affect the vigor of the root system and its aeration effectiveness.

This particular syndrome has been compared to a positive feed-back loop by Howes et al. (1981). It illustrates the complexity of the interactions among the plant responses themselves, and between the various aspects of the anaerobic root environment. The main features

of this model are as follows. The vigor and productivity of Spartina alterniflora are positively correlated with the substrate redox potential due to its interaction with root aeration (Linthurst, 1979; 1980; Mendelssohn and Seneca, 1980; Mendelssohn et al., 1981) because: 1) the lower redox levels represent an $O_2$ sink which overtaxes the plant's aeration system, and 2) phytotoxins accumulate to such a level that the roots' oxidizing power can no longer adequately ameliorate them (Teal and Kanwisher, 1961; Ponnamperuma, 1965; Carlson, 1980; Mendelssohn et al., 1981). As the $O_2$ demand supersedes supply the roots must rely more heavily on anaerobic respiraton and the vigor of the plant is adversely affected, and this lessens its capacity to oxidize the rhizosphere (Joshi et al., 1973; 1975).

## ROOT AERATION

Oxygen from above-ground organs may in some cases be sufficient for aerobic respiration of flooded roots. This is the most efficient adaptation in terms of energy production because aerobic respiration nets 36 moles of ATP (adenosine triphosphate) per mole of glucose as compared to the two ATP's yielded by lactic acid or alcohol fermentation (Lehninger, 1975). It is therefore not surprising that one major school of thought contends that the most important determinant of flood tolerance is the ability of a plant to aerate the roots under flooded conditions. One of the most well known workers associated with this philosophy is W. Armstrong. He stated,

> "The reliance on aerobic respiration for growth and the permanency of the anoxic soil environments clearly demonstrates the need for some form of ventilation in submerged plant organs; the presence of phytotoxins, many of which can be rendered harmless by direct oxidation or biological destruction, also indicates the desirability for ventilation (Armstrong, 1978 p.270)."

The importance of aeration is suggested by the fact that diffusion characteristics of roots have proven to be qualitative predictors of flood tolerance in certain cases. Jensen et al. (1967) measured the $O_2$ root permeability and diffusion rates of intact Hordeum vulgare, Oriza sativa, and Zea mays plants using $^{18}O$ analysis by mass spectrophotometry. From their determinations of longitudinal diffusional coefficients and surface permeability of $O_2$ in these three species, they developed a model to predict the partial pressure of $O_2$ as a function of distance along the root. Their predictions were in agreement with the known relative tolerances to anaerobiosis of these species: O. sativa > Z. mays > H. vulgare.

Intercellular Spaces

Oxygen movement within plants is dependent upon tissue having the property of relatively low diffusive resistance to gaseous diffusion. Aerenchyma is the most thoroughly studied tissue having this property, although it is not the only one. Aerenchyma is defined as, "Parenchyma tissue containing particularly large intercellular spaces

of schizogenous, lysigenous, or rhexigenous origin (Esau, 1977 p. 501).

Aerenchyma has long been recognized as an important adaptation to anaerobic environments. In 1921 Dunn demonstrated that aerenchyma formation could be induced in the roots of Zea mays and Triticum aestivum upon exposure to anaerobic conditons. Teal and Kanwisher (1966) reported that Spartina alterniflora had continuous aerenchyma from the leaves to the root tips, and that the aerenchyma was, "... sufficient to supply the aerobic respiratory needs of the roots as well as to aerate appreciably the reduced mud itself (Teal and Kanwisher, 1966 p. 355)." However, this claim has since been somewhat qualified because Spartina alterniflora must rely on anaerobic respiration under extremely reducing soil conditions (Mendelssohn et al., 1981). Williams and Barber (1961) also considered aerenchyma to be in excess of the amount needed for respiration and proposed the mechanical-cum-metabolic theory: aerenchyma was adaptive mainly in decreasing the $O_2$ demand and making the most efficient use of tissue for structural support. They also suggested that bouyancy and gas reservoir space were additional roles. Armstrong (1972) pointed out that while this theory was not necessarily invalid, aerenchyma was not likely to be present in excess because rhizosphere oxidation represented an important adaptation and $O_2$ sink.

Although the term "aerenchyma" is sometimes used in conjunction with woody species (e.g. Pereira and Kozlowski, 1977), their intercellular spaces are usually much less well-defined than those of

herbaceous species (Hook and Scholtens, 1978) and the term is usually avoided in this connection, presumably in deference to the "particularly large" criterion of the definition cited above (Esau, 1977). Two notable exceptions are mangrove (Scholander et al., 1955) and some species of Salix (Makarevic, 1956).

Nevertheless, the ability to aerate root systems with oxygen supplied from emergent organs through lenticels, aeration via intercellular spaces, and adventitious roots is generally thought to be of considerable adaptive importance to woody species under flooded soil conditions. Flooding usually induces these features in woody flood-tolerant species, but not in flood-intolerant species. This process has been described for several flood-tolerant tree species: Fraxinus pennsylvanica Marsh. (Sena Gomes and Kozlowski, 1980), Salix nigra, Ulmus americana, Populus deltoides (Pereira and Kozlowski, 1977), Nyssa sylvatica var. biflora, and Nyssa aquatica (Hook et al., 1970). Not only is this thought to be important because of the need for effective oxygen supply, but Chirkova and Gutman (1972) have demonstrated that potentially toxic substances such as ethanol, acetaldehyde, and ethylene can diffuse out of lenticels.

Hook et al. (1971) compared the anatomical, morphological and physiological responses of Nyssa sylvatica var. biflora seedlings that had been subjected to anaerobic conditions in the root environment with some that had been grown under aerobic conditions. They found that the seedlings subjected to anaerobic conditions responded by developing new roots which originated mainly from the tap root. This development was accompanied by the deterioraton of

the original secondary root system. The new roots had very little suberization, their endodermis was characterized by a less defined Casparian strip, and they were much more succulent as compared with their better-drained counterparts. The main functional difference in the new roots was that they were able to oxidize their rhizospheres in a solution of reduced indigo carmine dye while the non-flood acclimatized roots were not. Paradoxically, rhizosphere oxidaton occurred simultaneously with anaerobic respiraton.

Hook and his co-workers also demonstrated that a parraffin-lanolin coating on the stems of the previously flooded seedlings prevented this oxidation process and they deduced from this that the $O_2$ entered through the stem lenticels, and that the leaves made no discernable contribution. Armstrong (1968) found that in four flood-tolerant woody species; Myrica gale L., Salix atrocinerea Brot., Salix fragilis L., and Salix repens L.; oxygen supplied from the stem lenticels - not the leaves - was responsible for rhizosphere oxidation.

Adventitious roots are thought to be very important under flooded conditions because: 1) they apparently enhance $O_2$ transport (Sena Gomes and Kozlowski, 1980), 2) can tolerate high levels of $CO_2$ (Hook et al., 1971), and 3) efficiently absorb water (Sena Gomes and Kozlowski, 1980). Also, they are usually found in greatest abundance near the soil surface which is where $O_2$ content is highest and phytotoxins are lowest (Patrick and Delaune, 1972).

The importance of root aeration and adventitious roots in determining flood-tolerance has been disputed, however. Gill (1975)

removed the adventitious roots that formed on flooded Alnus glutinosa (L.) Gaertn. Although this treatment did result in a significant increase in adventitious root production, there was only a marginally significant and temporary decrease in leaf production, with no evident change in leaf diffusive resistance. This led him to doubt the putative adaptive value of flood-induced, adventitious roots in this species.

A recent study by Tripepi and Mitchell (1984b) indicated that neither adventitious roots nor lenticels are of primary significance in determining flood-tolerance. They compared four tree species: a flood-tolerant species with one less tolerant from two genera. The flood-tolerant species were Betula nigra L. and Acer rubrum L., and their less tolerant counterparts were B. pendula Roth and Acer saccharum Marsh. Experimental flooding induced hypertrophied lenticels in the A. rubrum and adventitious root formation on both flood-tolerant species. However, when the basal 14 cm of the stems were sealed so as to exclude oxygen, the root respiraton capacity was not further depressed in any of the four species. They suggested that the ability to utilize anaerobic respiration probably plays a primary role in flood tolerance, while root aeration plays a secondary role such as oxidation of reduced phytotoxins.

## Ethylene

Endogenously produced ethylene plays an essential role in the induction of aerenchyma formation. In 1955 Turkova reported that the effects of flooding on tomato plants were very similar to those of

ethylene treatment. This hormone has subsequently been studied intensively in flooding experiments. In 1972 Kawase found that submerged cuttings of herbaceous and woody species accumulated ethylene as a result of its lower solubility in water than in air ($1:1.7 \times 10^{-5}$). Kawase (1974) noted that ethylene apparently induced the formaton of aerenchyma and several other flooding symptoms: hypocotyl hypertrophy, adventitious root formation, epinasty, and chlorophyll breakdown. He later reported (Kawase, 1979) that ethylene stimulated cellulase activity, and that aerenchyma development could be induced in detached stem sections with exogenously applied cellulase. Later, the effects of exogenous ethylene on cellulase activiy and aerenchyma development was monitored in three herbaceous species (Kawase, 1981). Cellulase activity increased in each species as a result of ethylene treatment, and the magnitude of the response varied according to age and species. The increase in cellulase activity was accompanied by lysigenous aerenchyma development and was suppressed by $AgNO_3$, an ethylene antagonist. Based on these findings Kawase (1981) proposed that floodng caused an internal producton of ethylene, which stimulated higher cellulase activity, which in turn resulted in aerenchyma development. The production of 1-aminocyclopropane 1-carboxylic acid by the root tissue under anaerobic conditions is thought to trigger synthesis of endogenous ethylene (Yang, 1980).

In woody species, flood-induced, elevated rates of ethylene production result in cortex and lenticel hypertrophy, leaf senescense, negative geotropism of roots, and adventitious root

formation (Pereira and Kozlowski, 1977; Tang and Kozlowski, 1984b). Tang and Kozlowski (1984a) compared the responses of experimentally flooded seedlings of Fraxinus pennsylvanica (a flood-tolerant tree species) with unflooded controls. They compared the abaxial surface leaf diffusive resistance; leaf water potential; and ethylene release from stem pieces. The leaf diffusive resistance of the flooded seedlings increased dramatically during the first day of flooding but began to decline within five days; this decrease was well correlated with adventitious root formation. Stomatal closure was not the result of leaf dehydratrion because leaf water potential was higher in the flooded than drained individuals during the entire course of the experiment. Ethylene release was also stimulated by flooding and was correlated with lenticel hypertrophy and adventitious root formation. They interpreted this chain of events to be adaptive to flooding because: 1) the initial stomatal closure prevented leaf dehydration (likely to be brought about by deleterious effects to the root system by flooding), 2) then the ethylene-induced, adventitous roots permitted increased water absorbtion which permitted a reduction in leaf diffusive resistance.

Flooded soils are also a source of ethylene which can be absorbed by roots (Jackson and Campbell, 1975). For example, Smith and Restall (1971) found that under anaerobic soil conditions ethylene of microbial origin exceeded 20 ppm, a concentration 500 times in excess of that reported to induce waterlogging symptoms in tomato (Jackson and Campbell, 1976).

It is interesting from an ecological point of view that better

root aeration may result in a predisposition to dehydration under drought conditions. Hook and Brown (1972) investigated six tree species. They compared the pressure reduction required to draw air through submerged stem cuttings with: a) the bark (all tissue external to the vascular cambium) removed, and b) the bark intact. The vascular cambium of Nyssa aquatica and Fraxinus pennsylvanica, the two most flood-tolerant species, was permeable enough to permit aeration of the living cells of the root xylem via the lenticels. This suggested that the two hydrophytes could be supplied with $O_2$ from aerial sources, whereas the xylem of the mesophytes was supplied with $O_2$ via the transpiration stream, but as a consequence, "... excessive water loss by this pathway could result in desiccation of the cambium and newly formed xylem derivatives during periods of severe water stress (Hook and Brown, 1972 p. 310)." Keeley (1979) studied three ecotypes of Nyssa sylvatica, and he likewise found that drought tolerance was inversely related to flood tolerance.

Rhizosphere Oxidation

After the soil air is displaced with water, the oxygen is rapidly depleted. Subsequently, facultative then obligate anaerobic bacteria proliferate, and reduce a series of terminal electron acceptors. Nitrate is reduced when the eH (redox potential) reaches about 220 mV (all eH values in this discussion assume pH neutral soils). Manganic manganese is then reduced at about 200 mV, ferric iron at 120 mV, sulfate from -75 mV to -150 mV, and $CO_2$ between -250 to -300 mV (Gambrell and Patrick, 1978).

Survival under flooded conditions has frequently been linked to the ability to oxidize these compounds because the reduced compound of several of these redox couples is known to be phytotoxic. The oxidation of ferrous (reduced) iron has been reported as a flood adaptive trait in many species including: Phalaris sp., Phleum pratense L., Lotus corniculatus L. (Bartlett, 1961), Pinus contorta Dougl. (Sanderson and Armstrong, 1984), Erica cinerea (Jones and Etherington, 1970), Oriza sativa L. (Ponnamperuma et al., 1955; Green and Etherington, 1977), and Spartina alterniflora (Mendelssohn and Postek, 1982). Oxidation of manganous manganese and hydrogen sulphide have also been shown to be adaptive (Takijima, 1965; Jones and Etherington, 1970; Armstrong, 1972; Jones, 1972).

Although it has received relatively little attention, carbon dioxide is another potentially phytotoxic compound which tends to accumulate under flooded conditions (Ponnamperuma et al., 1965). Hook et al. (1970) were able to correlate soil $CO_2$ with poor growth of Nyssa sylvatica var. biflora and Nyssa aquatica, but unfortunately soil $O_2$ and $CO_2$ were inversely related and therefore the effect of $CO_2$ on growth could not be separated from the possible negative influence of low $O_2$ concentrations. Subsequently, Hook, Brown and Kormanik (1971) compared the effects of elevated $CO_2$ in a closed liquid culture system on Liquidambar styraciflua, and Nyssa sylvativa var. biflora. They found that the more flood tolerant species, N. sylvatica var. biflora, withstood elevated levels of $CO_2$ much better than L. styraciflua, which died within 15 days in 10% $CO_2$, and 10 days in 31% $CO_2$.

Nyssa sylvatica suffered no ill-effects from 15 days continuous treatment with either 2% or 10% $CO_2$; but at 31%, root development, height growth, oxygen uptake, and transpiration rate were retarded. Their conclusion was that the greater tolerance to elevated soil $CO_2$ was probably due to better rhizosphere oxidation.

Rhizosphere oxidation results in the formation of a coating or plaque on the root surface. This feature is mainly a consequence of the fact that the reduced forms of iron and manganese are soluble, but the oxidized forms are not (Gambrell and Patrick, 1978). Thus, when $Fe^{+2}$ and $Mn^{+2}$ are oxidized at the root surface they tend precipitate onto the root surface (Bacha and Hossner, 1977) and in some instances within epidermal and cortical cells (Green and Etherington, 1977; Chen et al., 1980). The amount of material deposited is generally regarded as a function of the amount of reduced Fe and Mn present (Bacha and Hossner, 1977) and the oxidizing capacity of the roots (Bartlett, 1961; Mendelssohn and Postek, 1982). Other factors such as pH and the presence of chelators can also be important (Taylor et al., 1984). It has been suggested that the reason iron is usually deposited in greater quantities than Mn is because Fe oxidation buffers the rhizosphere eH at a lower value than that at which Mn oxidaton takes place, and because of the much greater abundance of Fe than Mn generally present in soil (Mendelssohn and Postek, 1982).

## ANAEROBIC RESPIRATION

The term "anaerobic respiration" focuses on the plant responses that occur as a result of the depletion of oxygen in the root environment. The most extensively investigated aspects of this phenomenon are the enzymes and associated metabolic products known to be independent of oxygen. These plant responses have been described under experimental conditions in which the air of the root environment has been displaced with water or with an oxygen-free gas, and in solution culture from which the dissolved oxygen has been purged. Figure 1. illustrates the major anaerobic metabolic pathways.

It has been suggested by Davies et al. (1974) that anaerobic respiration is maladaptive because it will net only two moles of ATP per mole of glucose during ethanol and lactate formation, and in the case of malate accumulation it will not yield any ATP: whereas aerobic respiration will net 36 moles of ATP per mole of glucose utilized. Also, it has been argued that at the root tips, where respiration is highest, the tissue is so densely packed that anaerobic respiration is necessary even in well aerated soils (Crawford, 1978b). However, anaerobic respiration has also been demonstrated among plants considered to be flood tolerant, e.g. Oriza sativa (Bertani et al., 1980), and Spartina alterniflora (Mendelssohn et al., 1981). These points have stimulated inquiry into possible metabolic adaptations to flooding.

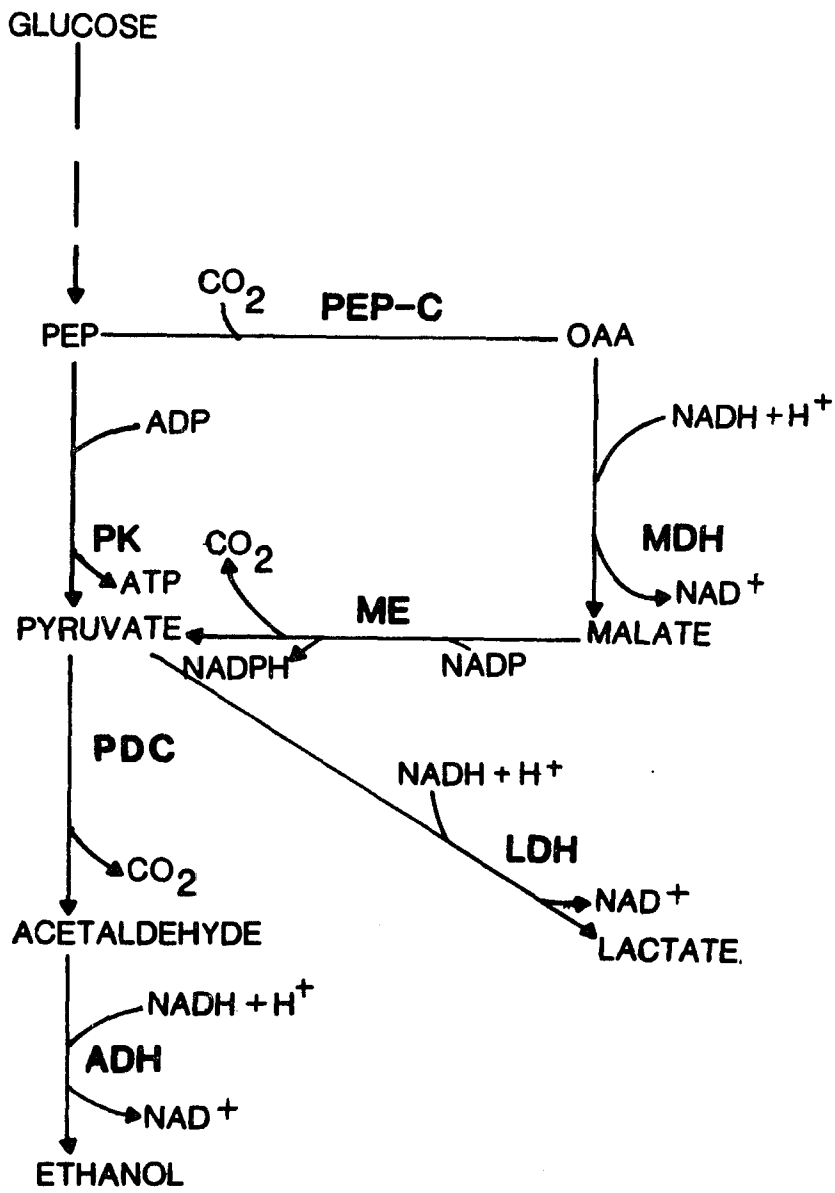


Figure 1. Schematic diagram of some possible metabolic processes occurring during anaerobiosis. Enzymes are PEP-C (phosphoenol-pryruvate carboxylase), MDH (malate dehydrogenase), ME (NADP-malic enzyme), LDH (lactic dehydrogenase), PDC (pyruvate decarboxylase), ADH (alcohol dehydrogenase), and PK (pyruvate kinase). OAA is oxaloacetic acid, and PEP is phosphoenolpyruvate.

Metabolic Switch Hypothesis: Pro

In 1971 McManmon and Crawford proposed a theory whereby flood-tolerant plants avoid an accumulation of ethanol, which they assumed to be autotoxic. They suggested that under anaerobic conditions the roots of flood-intolerants would accumulate acetaldehyde and ethanol due to the induction of ADH (alcohol dehydrogenase) activity and a reduction in its Km value. Flood tolerants, however, would not respond in this way. Therefore, acetaldehyde and ethanol would not accumulate. They hypothesized that a key factor in this metabolic adaptaton would be the absence of ME (NADP-malic enzyme). Because ME is missing, malate is not converted to pyruvate and ethanol production is precluded; nontoxic malate accumulates instead (see Fig.1). However, there is no net gain of ATP associated with malate synthesis.

In this scheme, PEP (phosphoenolpyruvate) is carboxylated via PEP carboxylase to oxaloacetic acid (see Fig. 1), which is then converted to malate with the regeneration of NAD (nicatinomide adenine dinucleotide) via MDH (malate dehydrogenase). The production of NAD is essential for the continuation of glycolysis, the common denominator of all respiration. It is also interesting to note that NADH is possibly an activator of pyruvate decarboxylase, the rate limiting enzyme in ethanol producton (John and Greenway, 1976; Chang et al., 1983), whereas NAD is likely inhibitory (John and Greenway, 1976).

Crawford and his co-workers have supplied considerable evidence in support of the metabolic switch theory. In one study, eleven species

were determined to be flood-tolerant or intolerant on the basis of dry weight gain or loss, respectively, under experimental flooding (Crawford and Tyler, 1972). Anaerobic respiration is usually concommitant with physical degeneration in flood-intolerant plants, but not with flood-tolerants (Crawford and Tyler, 1972). The ratio of malate content before flooding to the malate content after flooding was less than one for all flood-tolerants, and greater than one for flood-intolerants.

McManmon and Crawford (1971) demonstrated that out of 19 species flooded in sand culture for one month, the flood-tolerants had a decrease or very small increase in ADH activity; the flood-intolerants all had a relatively large increase. They singled out eight species in which the ME activity was measured before and after one month of flooding. Under both conditions the ME activity of the flood-tolerants was not detectable, while flood-intolerants all had measurable activity.

Crawford (1972) also pointed out that the sap of Betula pubescens, a flood-tolerant tree, had a malic acid content far in excess of ethanol, especially under the wettest conditions represented. He stated that the malic acid could possibly be used for synthetic processes in the aerial part of the plant, and "... the net result would be a gain of carbon to the plant from carbon dioxide fixation in the roots (Crawford, 1972 p. 315)." The glycerol content of Alnus incana, another flood-intolerant tree species, rose considerably over an eight day flooding period. The glycerol was argued to be analogous to malate. Also, a list of other possible nontoxic

alternatives to ethanol included lactic acid, pyruvic acid, succinic acid, glycerol, shikimic acid, glycoloc acid, glyoxalic acid, alpha-ketoglutaric acid, alanine, ethylene, gamma-amino butyric acid, glutamic acid, serine, and proline (Crawford, 1972).

Other workers' data have also supported the metabolic switch theory. Marshall et al. (1973) studied ADH isoenzyme varients specified by two ADH alleles in Zea mays. In an experiment using progeny from a single, self-fertilized, heterozygous parent, individuals homozygous for the less active form of ADH were more flood-tolerant than those homozygous for the more active form, while heterozygous plants were intermediately tolerant. This suggested that the less active form of ADH conveyed an adaptive advantage to flooding due to decreased ethanol production. Similar results have also been reported from work with Helianthus annuus (Diedenhofen, 1977).

Linhart and Baker (1973) found that within a single population of Veronica peregrina, differential accumulation of malate under flooded conditions corresponded to microhabitat distribution. They collected seeds from individuals near the center and near the edge of a temporary pool. The center was flooded for over two months of the year whereas the edge was usually flooded only for very short periods. The plants grown from seed collected near the center accumulated a significantly larger amount of malate in the root tissue under the flooding treatment than those from the edge. This indicated that increased flood-tolerance resulted from a decrease in ME activity.

Mendelssohn et al. (1981) studied Spartina alterniflora along a continuous transect from a streamside zone of vigorous growth, through an inland area of moderate growth, to a die-back area of very poor growth. They found that sufficient $O_2$ did not reach the roots of inland S. alterniflora to preclude anaerobic metabolism as previously thought (Teal and Kanwisher, 1966). Furthermore, the roots of the streamside plants, accustomed to mostly moderately anaerobic conditions, responded to these conditions with an increase in the root malate content. Roots in the inland area respired primarily by alcoholic fermentation, but the amount of ethanol in the tissue was relatively small because of diffusion out of the roots. Roots from the die-back region also depended on alcoholic fermentation, but apparently the toxic nature of the substrate resulted in poorer plant vigor and intermediate ADH levels. Thus, flood tolerant S. alterniflora responded to moderate anaerobiosis (within the streamside area) by accumulating malate as per the metabolic switch theory.

An apparent connecton between ethanol production and intolerance to flooding was reported in a comparative study involving Medicago sativa L. (alfalfa) and a more flood tolerant species, Lotus corniculatus (trefoil) (Barta, 1984). The ethanol excreted via the transpiration stream was measured and none was detectable from L. corniculata, but M. sativa transpired significant amounts which peaked just before injury symptoms were observed. Ethanol excretion and accumulation from the roots of both species were also compared. The roots of both species produced elevated amounts upon flooding,

but M. sativa synthesized and accumulated more than the L. corniculatus. Also, the ADH Km of both species increased but that of L. corniculatus increased to the greater extent. These relative differences were consistent with Crawford's metabolic theory.

Metabolic Switch Hypothesis: Con

Davies et al. (1974b) investigated the properties of ME in the roots of 38 species. Malic enzyme activity was detected in all of the species examined including the four flood-tolerants in which McManmon and Crawford (1971) had found no activity. Davies et al. argued that due to the fact that ME was present in flood-tolerant species, the metabolic switch theory was untenable. Similar findings and conclusions were also reported by Chirkova et al. (1974) and by Smith and ap Rees (1979). Davies proposed that the role of malate and ME was that of a pH stat. Malate is a relatively strong acid. Therefore, since, under physiological conditions, PEP carboxylase (see Figure 1) has an alkyline pH optimum, while ME has an acidic pH optimum, these two enzymes buffer cytoplasmic pH changes toward neutrality (Davies, 1977; 1980).

Hook et al. (1971) found that after a one month flooding experiment, $N_2$ incubation of excised roots of Nyssa sylvatica var. biflora resulted in higher levels in roots that had been flooded as compared to those that had not been. Anaerobic root conditions resulted in increased ADH activity in this flood tolerant tree species. McManmon and Crawford (1971) had argued that an increase would be characteristic of intolerant species.

Keeley (1979) studied the flood-induced responses of three Nyssa sylvatica ecotypes: swamp, floodplain, and upland, to a one-year flooding period. As their names imply, the flood-tolerance of these trees decreases in the order listed above. During the first week of flooding all three accelerated alcoholic fermentation. After one month the upland ecotype had the lowest rate of alcoholic fermentation, lowest ADH activity, and the lowest malic acid accumulation; while the swamp the swamp ecotype had high alcohol production, high ADH activity, and high malic acid concentraton. After one year the swamp ecotype had a low rate of alcoholic fermentation but a high malic acid content. Keeley proposed that the malate served to counterbalance potentially toxic cations such as reduced iron and manganese rather than as an alternative to ethanol. This role of malate has been suggested by several other authors as well (e.g. Raven and Smith, 1974; Mathys, 1977; Keeley, 1978; 1979). Keeley attributed the decrease in ethanol production in the swamp ecotype to the replacement of the original secondary root system with one that was better aerated. The resulting aeraton efficiency of these ecotypes corresponded to their degree of flood tolerance and inversely with the uptake of iron and manganese.

Tripepi and Mitchell (1984b) investigated the responses of two birch species, Betula nigra L. and Betula pendula Roth., and two maple species, Acer rubrum L. and Acer saccharum Marsh., to controlled flooding conditions over a 30 day period. They found that hypoxia resulted in reduced malate content of both species, and alcohol fermentaton was maintained at higher rates over a longer time

period in B. nigra, the more tolerant of the two species. Both of these phenomena are contradictory to the metabolic theory of flood tolerance a la Crawford. They also reported that even though ADH activity was greatly elevated under flooding, ethanol could not be detected in either the root extracts or the nutrient solutions in which the experiments were conducted. After 18 days of anoxia, the ATP content of the B. nigra seedlings were 23% of the aerated controls, while those of B. pendula were 8%. This difference was consistent with the higher ADH activity of the more flood-tolerant species. Thus, ATP formation via alcohol fermentation was suggested as the major determinant of flood tolerance.

Rumpho and Kennedy (1981) have shown that Echinochloa crus-galli seedlings subjected to a completely anoxic environment increased their ME activity five-fold over a seven day period. Their ADH activity and ethanol production increased, but neither malate nor lactate were produced in appreciable amounts. These data are in contradiction with the metabolic switch theory. It was found that eighty-five per cent of the ethanol produced was excreted. These results corresponded closely with those of Bertani et al. (1980), who subjected Oryza sativa seedlings to complete anoxia. The seedlings produced $CO_2$ and ethanol in equivalent amounts indicating that they had adopted a completely alcoholic fermentation. Ninety-eight per cent of the ethanol was excreted. Thus, increased alocholic fermentation coupled with the ability to excrete large amounts of ethanol were determined to be key adaptations for these two species (Bertani et al., 1980; Rumpho and Kennedy, 1981), E. crus-galli and

O. sativa, which are the only two species known to be able to survive total anoxia for any significant length of time (Rumpho and Kennedy, 1981).

Crawford (1978) modified the metabolic switch theory in view of the reports of ME found in flood-tolerant plants. He suggested that ME, although not absent, was nevertheless inhibited in flood-tolerant plants. This implied that some alcohol would likely be produced under anaerobic conditions even by flood-tolerant plants. He further qualified his theory by stating that ethanol may not accumulate to toxic levels in species such as Oryza sativa, which could excrete large amounts through their roots. Therefore the metabolic switch theory would not apply in these cases.

These modifications of the metabolic switch theory did not abate the controversy. The assumption that ethanol is phytotoxic at physiological concentrations came under attack. Jackson et al. (1982) examined the effects of ethanol on flood-intolerant Pisum sativa at the flowering and fruiting stage, which is when it is most vulnerable to flooding damage. They found that,

> "... ethanol in aerobic or anaerobic nutrient solution at similar concentrations to those we found in flooded soil (up to 3.9 mol $m^{-3}$) or in the xylem sap of flooded pea plants (up to 2.1 mol $m^{-3}$) caused no injury. One hundred times these concentrations gave little extra effect and failed to stimulate flooding injury (Jackson et al., 1982 p. 163)."

This finding in conjunction with a thorough review of relevant literature led these authors to doubt that ethanol toxicity and ADH responses under flooded conditions were actually significant determinants of flood tolerance. Likewise, Rumpho and Kennedy (1983) were unable to induce injury in germinating seeds of Echinochloa crus-galli with the application of 45 times the endogenous amount of ethanol.

Davies (1980) is among those who argued that ethanol is unlikely to build up to toxic levels within plant cells. He cited Davis (1958) who noted that the end products of metabolism such as urea and ethanol are uncharged and therefore readily eliminated from cells, unlike metabolic intermediates which are charged and tend to be retained. Davies stated that in some plants ethanol is transported to aerobic tissues where it is oxidized, used in gluconeogenesis, or diffuses out of the plant. He concluded that the correlation between flood intolerance and high ethanol levels is "... to be seen as a consequence, rather than the cause, of cell and tissue damage (Davies, 1980 p. 602).

Perhaps the evidence most damaging to the metabolic switch hypothesis thus far is the apparent role that ethanolic fermentation plays in preventing cytoplasmic acidosis under anaerobic conditions. No precise measurements of cytoplasmic pH in the living cells of higher plants were available prior to the application of nuclear magnetic resonance (NMR) spectroscopy to this problem. Justin Roberts and his coworkers have demonstrated that this technique can

be used to monitor the pH in the cytoplasm and vacuole of higher plant cells (Roberts et al., 1980; Roberts, 1984), and have utilized NMR in several physiological investigations, including the role of ethanolic fermentation in cytoplasmic pH regulation during anaerobiosis. One major finding was that the release of cytoplasmic $CO_2$ from the roots during fermentation (see Fig. 1) buffers cytoplasmic pH, and that if the rate of $CO_2$ escape is lessened by i) a deficiency of ADH, ii) exposure to external $CO_2$, or iii) a decreased flow of nutrient solution around the roots, then cytoplasmic acidosis and root mortality will ensue (Roberts, 1984a). This suggests that alcoholic fermentation, rather than being a detrimental process as suggested by the metabolic switch hypothesis, is actually beneficial because under anaerobic conditions it buffers cytoplasmic pH and maintains ATP levels (Roberts, 1984a; 1984b). This view of the role of ethanolic fermentation in cytoplasmic pH buffering agrees with that of Davies (1974b; 1977).

## SUMMARY

When a soil is flooded the oxygen is rapidly depleted, alternate electron acceptors become reduced, the redox potential decreases, and potentially toxic compounds tend to accumulate (Ponnamperuma, 1965; Gambrell et al., 1977; Gambrell and Patrick, 1978). There are several complicating plant-soil interactions that are characteristic of flooded systems, e.g., rhizosphere oxidation, and plant and soil produced ethylene, ethanol, and $CO_2$. In addition, the

interpretation of field studies may be confounded by the effects of competition because a flood adapted species may encounter less inter-specific competition and thus have access to more resources in waterlogged environments (Menges and Waller, 1983).

The importance of root aeration as an adaptation to flooding is indicated by the following: 1) waterlogged soil conditions induce the formation of tissue that is less resistant to gaseous aeration in flood-tolerant plants, 2) root aeration permits aerobic respiration which is more energy efficient than anaerobic respiration, 3) root aeration permits the amelioration of reduced phytoxins which often accumulate in flooded soil conditions. In herbaceous species aerenchyma represents an energy efficient flood adaptation that can help avoid anaerobiosis altogether (Hochanchka and Somero, 1973). It allows less resistance to $O_2$ movement for respiring cells (Armstrong, 1978) and decreases the amount of respiring tissue while still supplying structural support (Williams and Barber, 1961). In woody species, while aerenchyma rarely develops, the movement of air through intercellular spaces and across the vascular cambium may be essential to survival under prolonged flooding (Hook and Brown, 1972).

Rhizosphere oxidation ameliorates soil toxins such as hydrogen sulphide, and reduced iron and manganese (Armstrong, 1972). It is affected by age (Kawase, 1981), genotype (Keeley, 1979), acclimatization (Hook et al., 1971), and substrate redox potential (Mendelssohn and Postek, 1982). Root-coatings form on root surfaces to an extent that is mainly dependent upon the amounts of reduced Fe

and Mn in the soil and the oxidizing capacity of the roots. Roots are capable of simultaneously respiring anaerobically and oxidizing their rhizospheres (Hook et al., 1971; Lambers, 1976).

Many structural modifications induced by flooding are thought to be mediated by ethylene. Ethylene can induce: lysigenous parenchyma via cellulase activation (Kawase, 1981), adventitious root formation (Jackson et al., 1981), dry weight reduction in roots and shoots, reduced seminal root length and increased diameter, increased lateral root length and root hair proliferation (Crossett and Campbell, 1975), and possibly increased endodermis water resistance (Hunt et al., 1981). In some flood-tolerant tree species, increased root resistance has been offset by stomatal closure until adventitious roots form, which are better able to absorb water under flooded conditions (Sena Gomes and Kozlowski, 1980).

Ethanol can be toxic due to its solubilizing effect on cell membranes (Kiyosawa, 1975), but whether or not it builds up to toxic levels in plant cells and tissues is debatable (Davies, 1980; Barclay and Crawford, 1981; Jackson et al., 1982). Based on the assumption that it is autotoxic, a theory of metabolic adaptation was proposed whereby there is a switch from ethanol to nontoxic malate production (McManmon and Crawford, 1971). Other roles for malate in anaerobiosis have also been proposed: to counterbalance cations which may be absorbed in excess of anions in reduced soils (Keeley, 1978), and also as a component of the cytoplasmic pH control system (Davies, 1980). Ethanol production has also been ascribed two major roles in flood tolerance: 1) cytoplasmic pH buffering during anaerobiosis

through $CO_2$ evolution (Davies, 1974b; 1977; Roberts, 1984a; 1984b), and ATP production (Mendelssohn et al., 1981; Roberts, 1984a; 1984b; Tripepi and Mitchell, 1984a).

Generalizations in this area are difficult to make. It is apparent that genotype, age, vigor, environment, and probably other factors as well, interact to produce an integrated response resulting in some degree of flood tolerance (Hook and Brown, 1973; Mendelssohn et al., 1981). Most experiments of plant responses are of a fairly short duration. Keeley's (1979) work is an exception to this generalization. His data were collected over a one-year period. The results showed that flooding responses are dynamic, and that short-term adaptations may be very different than long-term, even within the same ecotype. Another difficulty is that standardized methods of comparisons are virtually non-existent. Results from plants subjected to various anaerobic root environments, e.g. nutrient solutions bubbled with either air or nitrogen, flooded sand cultures, flooded soil, and anaerobic gaseous environments are sometimes compared. The underlying assumption is that the roots are responding primarily to anoxia per se or some sort of generic "flooding stress"; however, numerous factors such as pH, Eh, and $CO_2$ levels can be important.

Flooding results in many concomitant changes in soil chemistry and a wide array of potential problems for plant roots. There are likewise many adaptations to waterlogged conditions. As a result, the specific cause-effect relationships of flood-induced responses are not completly understood. Although much progress has been made

in this area, many challenges remain.

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# CHAPTER THREE

# EXTRACTION OF ROOT ALCOHOL DEHYDROGENASE FROM GREEN ASH AND WATER OAK

ABSTRACT. The extraction of alcohol dehydrogenase (ADH) (E.C. 1.1.1.1) from the roots of water oak (Quercus nigra L.) and green ash (Fraxinus pennsylvanica Marsh.) was optimized. Protective additives were tested for effectiveness in preventing enzyme inhibition by polyphenolic compounds during extraction. Although both ash and oak roots contained substantial amounts of phenolic compounds, the differences in the composition and amount of their constituents resulted in a different pattern of response to the additives tested. A buffer solution with the protective agents dithiothreitol (DTT) and soluble polyvinylpyrrolidone resulted in the highest recovery of ADH and total soluble protein from oak roots which contained large amounts of tannins. The additives DTT and insoluble polyvinylpolypyrolidone were most effective in the extraction of ADH and protein from ash roots which contained substantial amounts of non-tannic phenolic compounds. Cryodessication prior to extraction enhanced the recovery of ADH from the oak roots and, to a lesser extent, from the ash. The results of this study demonstrated that the method used to extract ADH from plant roots containing different types of phenolic compounds must be optimized for each plant species.

# INTRODUCTION

Variations in root adaptations to flooding are responsible for differences in plant distribution at the species (Crawford and Tyler, 1969), ecotype (Keeley, 1979), and intra-population (Linhart and Baker, 1973) levels. Root metabolic responses can indicate how well and by which mechanisms a plant is adapted to the aerobic environment of flooded soils. Root enzyme activities have thus been measured to determine the relative importance of various metabolic pathways during anaerobiosis (e.g. Wignarajah et al., 1976; Smith and ApRees, 1979a; 1979b; Mendelssohn et al., 1981; Tripepi and Mitchell, 1984). The accurate determination of enzyme activities in plant roots can be seriously hampered, however, by polyphenolic compounds since their presence in tissue extracts can cause protein precipitation and enzyme inhibition (Loomis and Battaile, 1966; Loomis, 1974). Although studies comparing root enzyme activity among different flood-tolerant and flood-intolerant species have been frequently made (Wignarajah et al., 1976; Smith and ApRees, 1979b; Tripepi and Mitchell, 1984; Taylor, 1942; McManmon and Crawford, 1971; Jenkin and ApRees, 1983), few have taken into account the effect of phenolic compounds on the activity of the enzymes measured.

The isolation and complete recovery of enzymes from plant tissue is often difficult primarily due to the inevitable release of secondary compounds from cell walls and vacuoles. This results in the partial or total inactivation of the enzymes under investigation (Loomis and Battaile, 1966; Anderson, 1968). The standard approach

to the extraction of enzymes from plant tissues has been to prevent the interaction of phenolic compounds with the enzymes by the addition of protective agents to the extraction solution. The chemistry of the various methods which have been used to circumvent the enzyme inhibition caused by phenolic compounds has been reviewed in detail by several authors including Loomis and Battaile (1966), Anderson (1968), and Rhodes (1977). Briefly stated, most phenolic interferences fall into one of two categories: quinones or tannins. Inactivation by quinones is often ameliorated through the use of thiols or other reducing agents. Thiols rapidly convert quinones into thioethers, thereby preventing their accumulation (Loomis amd Battaile, 1966). Tannins are commonly dealt with by the addition of an excess of a competitive polymeric substrate such as PVP (polyvinylpyrrolidone).

The amount and composition of phenolic compounds in plants may vary with species, age, tissue type, and environment (Ribereau-Gayon, 1972). Tree species have long been known to be a source of phenolic compounds. For this reason, the two tree species used in this investigation, water oak and green ash were thought likely to have high contents of potential inhibitors of ADH (alcohol dehydrogenase), the primary enzyme measured in studies of anaerobic root metabolism. The major objectives of this study were to 1) demonstrate the importance of optimization of enzyme extraction methods for plant tissues containing phenolic compounds and 2) provide broad guidelines for the extraction of ADH from the roots of plant species containing tannic and non-tannic phenolic compounds.

# MATERIALS AND METHODS

## Plant Material:

One year old seedlings of green ash, Fraxinus pennsylvanica Marsh., and water oak, Quercus nigra L., were obtained from the Louisiana Forestry Commission and potted in 20 X 20 cm plastic pots containing Jiffy Mix Plus (a commercial potting mixture containing nutrients, Jiffy Products of America, West Chicago, Illinois). The seedlings were allowed to grow in a greenhouse for eleven months where the temperature ranged from 20°C (night) to 30°C (day) and the light intensity at midday was ca. 1500 $\mu$mol $m^{-2}$ $s^{-1}$. The two year old seedlings of each group were flooded such that the potting medium was completely submerged for three days. The roots were harvested by washing them free of the potting mixture and then rinsing with deionized water. Living, i.e. turgid and structurally intact, roots were quickly separated from the dead roots and debris and placed in plastic bags on ice. Different sets of seedlings were harvested for each experimental comparison, i.e. preliminary survey of extractants, comparison of PVP(P) [polyvinyl(poly)pyrrolidone] concentration and pH effects, and comparison of fresh versus freeze-dried tissue. The preliminary survey of extractants and assay buffer pH comparison were each conducted three times with roots from individual seedlings. The remainder of the experimental comparisons were conducted with roots from several seedlings which were mixed so

that a composite sample for each species was obtained; these experiments were replicated three or four times. The fresh root tissue was placed on ice and extracted within 30 min after collection as described below. An aliquot from each of the composite oak and ash roots was also freeze-dried in a LabConco Freeze-Dryer 5 and ground in a Wiley-Mill (# 60 mesh sieve) for use in the measurement of phenolic content, tannin content, and ADH standard recovery.

Enzyme Extraction and Analysis:

The root tissue (0.5 g fr. wt) from each complete sample was cut into 1-2 mm sections, immediately added to 5.0 ml of extraction solution, and homogenized in a mortar and pestle with a small amount of acid-washed sand added to the extraction solution to facilitate grinding. The homogenate was centrifuged at 20,000 g for 30 min and the supernatant assayed within 1 h. All were prepared and maintained at 2-4$^{o}$C until they were assayed for alcohol dehydrogenase (E.C. 1.1.1.1) at 30$^{o}$C in the reaction mixture described as follows: 2.8 ml total volume, 0.1 ml sample extract, 5.4 mM $MgCl_2$, 0.26 mM NADH, and 0.40 mM acetaldehyde in 14 mM Tris buffer, pH 7.3 Enzyme activity was measured by following the oxidation of NADH (nicotinamide adenine dinucleotide reduced form) in the reaction cuvette against a blank containing all compounds except acetaldehyde at a wavelength of 340 nm in a Beckman Model 35 spectrophotometer.

Protein Analysis:

Total soluble protein was determined according to Bradford (1976) on the supernatant from each of the sample extractions within 1 h after the assay. Each sample was read against a blank containing the appropriate extraction buffer.

Preliminary Survey of Extraction Additives:

Several protective additives were tested initially to identify those which were most effective for ash or oak. Root tissue was extracted (as described above) in each of the solutions listed in Table 1.

Comparison of Six Extraction Solutions:

Based on the results of the preliminary survey, the following six extraction solutions were tested for effectiveness in extraction and recovery of alcohol dehydrogenase (ADH) from ash and oak roots: 1) 100 mM Tris buffer (pH 7.3) which contained 5 mM $MgCl_2$ and designated as "T", 2) 100 mM Tris buffer (pH 7.3) which contained 5 mM $MgCl_2$ and 20 mM DTT and designated as "TD", 3) 100 mM Tris buffer (pH 7.3) which contained 5 mM $MgCl_2$ and 10% (w/v) soluble PVP and designated as "TP", 4) 100 mM Tris buffer (pH 7.3) which contained 5 $MgCl_2$, 10% (w/v) PVP, and 20 mM DTT and designated as "TPD", 5) 100 mM Tris buffer (pH 7.3) which contained 5 mM $MgCl_2$ and 10% (w/v) of insoluble PVPP (polyvinylpolypyrrolidone) and designated as "TPP", 6) 100 mM Tris buffer (pH 7.3) which contained 5 mM $MgCl_2$, 10% PVPP (w/v), and 20 mM DTT and designated "TPPD".

Table 1. List of solutions tested for the extraction of alcohol dehydrogenase (ADH) from green ash and water oak roots.

| Extractant number | description of solution |
|---|---|
| #1 | 100 mM Tris buffer with 5 mM $MgCL_2$ (pH 7.3). |
| #2 | #1 + 2% (w/v) bovine serum albumin (BSA). |
| #3 | #1 + 20 mM ascorbic acid. |
| #4 | #1 + 20 mM cysteine. |
| #5 | #1 + 20 mM dithiothreitol (DTT). |
| #6 | #1 + 20 mM mercaptoethanol (ME). |
| #7 | #1 + 10% (w/v) soluble polyvinylpyrrolidone (PVP). |
| #8 | #7 + 2% (w/v) BSA. |
| #9 | #7 + 20 mM ascorbic acid. |
| #10 | #7 + 20 mM cysteine. |
| #11 | #7 + 20 mM DTT. |
| #12 | #7 + 20 mM ME. |

ADH Recovery:

A known amount of standard ADH was added to freeze-dried tissue extractions to determine the effect of each of the six extraction solutions described above on the per cent recovery of an internal standard of ADH in the presence of oak or ash root phenolics. The freeze-dried, ground root tissue (0.1 g dry wt) was added to 5 ml of each of the six extraction solutions which contained a known amount of standard ADH and vortexed for 15 sec. Recoveries of standard ADH were also determined with fresh tissue extracts by addition of a known amount of ADH to 5 ml of each of the six extraction solutions prior to grinding 0.5 g fr. wt of the oak or ash roots. The extracts were centrifuged and assayed for ADH as described above.

Optimization of Extraction and Assay Conditions:

The optimum pH and adsorbent concentration for the extraction of ADH from ash and oak roots was determined. Ash and oak roots were extracted in 100 mM MES buffer at pH 6.0 and in 100 mM Tris buffer at pH 7.5 and 9.0; all solutions contained 6 % (w/v) PVP or PVPP and 20 mM DTT. Concentrations of PVP or PVPP (w/v) tested were 2, 6, and 10%. The pH of the assay buffer was also varied as follows: 40 mM MES for pH 5.5 and 6.0; 40 mM MOPS for pH 6.5 and 7.0; and 40 mM Tris for 7.5, 8.0, 8.5, and 9.0; all other assay conditions were described as above.

Total Phenolic Content:

Phenolic content was determined by the Folin-Denis Assay (Ribereau-Gayon, 1972; Swain and Hillis, 1959). Freeze-dried, ground oak and ash root tissue (0.05 g) was extracted in 4 ml of 50% aqueous methanol. A 0.10 ml aliquot of extract was reacted with 0.20 ml of Folin-Denis reagent in a total volume of 3.45 ml and absorbance at 725 nm read one h after addition of a saturated solution of sodium carbonate. A calibration curve was constructed using tannic acid.

Tannin Content:

Hydrolyzable tannins were determined by a modified iodate technique (Bate-Smith, 1977). Aliquots (2.5 ml) from the oak and ash extracts used for phenolic content determination were cooled on ice; absorbances were read at 550 nm, 40 min after addition of 0.1 ml of a saturated $KIO_4$ solution. A calibration curve was constructed using tannic acid.

Condensed tannins were determined by the proanthocyanidin method (Bate-Smith, 1975). Aliquots (0.4 ml) of the extracts were added to 3 ml of 80% butanol-HCl containing 15.4% ferrous sulfate and heated at 97$^{o}$C for 15 min. Absorbance was read against unheated blanks at 550 nm. A calibration curve was constructed using commercial bisulfited quebracho (Pilar River Plate Corp., Newark, New Jersey).

ADH Activity in Fresh and Freeze-dried Tissue:

The effect of freeze-drying on ADH extraction effeciency was determined with oak and ash roots. Fresh roots (0.5 g fr. wt) were

collected as before, mixed, and divided into two aliquots. One aliquot was immediately extracted in 5 ml of the appropriate extraction solution as described above. The other aliquot was placed in ca. 20 ml of deionized water in a plastic bag (the water forms a protective layer of ice which prevents tissue melt-back during handling), frozen in liquid nitrogen, and freeze-dried (Mendelssohn and McKee, 1981). The freeze-dried, ground (#60 mesh sieve) roots (0.05 g dry wt) were extracted in 5 ml of the same extraction solution by vortexing for 15 sec. Extracts were centrifuged and assayed as described above.

## RESULTS AND DISCUSSION

A preliminary examination of protective additives (Table 1) demonstrated that PVP, DTT, and their combination were equal to or better than agents such as bovine serum albumin, ascorbic acid, mercaptoethanol, and cysteine in preventing the inhibition of ADH in ash and oak root extracts (Table 2). The inclusion of DTT alone produced the highest ADH activity in the ash roots of all the additives tested, but had little effect in the oak extracts. Other reducing agents such as mercaptoethanol and ascorbic acid did not prevent ADH inhibition in the ash extracts as well as DTT. A combination of PVP and a reducing agent such as DTT was most effective with the oak roots. Bovine serum albumin was somewhat effective with both tree species, but did not prevent ADH inhibition

Table 2. Preliminary comparison of extraction solution efficiencies for alcohol dehydrogenase measurements (umoles $g^{-1}$ f wt $h^{-1}$) of green ash and water oak roots*.

| Extractant** | ash mean ± (standard error) | oak mean ± (standard error) |
|---|---|---|
| #1, Tris | 5 ± (1) | 0 ± (0) |
| #2, Tris + BSA | 96 ± (5) | 47 ± (19) |
| #3, Tris + ascorbate | 35 ± (13) | 0 ± (0) |
| #4, Tris + cysteine | 0 ± (0) | 2 ± (2) |
| #5, Tris + DTT | 250 ± (32) | 8 ± (8) |
| #6, Tris + ME | 121 ± (18) | 10 ± (10) |
| #7, Tris + PVP | 6 ± (1) | 76 ± (43) |
| #8, Tris + PVP + BSA | 51 ± (8) | 138 ± (26) |
| #9, Tris + PVP + ascorbate | 104 ± (12) | 191 ± (20) |
| #10, Tris + PVP + cysteine | 1 ± (1) | 2 ± (2) |
| #11, Tris + PVP + DTT | 221 ± (58) | 203 ± (20) |
| #12, Tris + PVP + ME | 187 + (47) | 154 + (6) |

* Green ash were flooded for 3 days, water oak for 6.

** Extractants are described in Table 1.

as well as did the PVP for oak or DTT for ash. The inclusion of cysteine had little effect on preventing ADH inhibition in either species, even in combination with PVP.

Both ash and oak roots contained phenolic compounds (Table 3). The oak roots contained both condensed and hydrolyzable tannins, while the ash roots did not (Table 3). The differences in the composition and amount of their polyphenolic constituents resulted in a different pattern of ADH response to the six combinations of additives which included DTT, PVP, and insoluble PVPP (Figs. 1 & 2). The results indicated that the inhibition of ADH by tannins was more important in the oak root extracts, while non-tannic phenolics were responsible for the inhibition in the ash (Table 3; Figs. 1 & 2).

In addition, the inhibition of activity of added internal ADH standards in the oak extracts was greatest in those solutions which did not contain PVP (Fig. 2). PVP has been shown to reduce the interference by tannins (Loomis and Battaile, 1966) and other higher molecular weight phenolic compounds (Anderson, 1968). When ADH standards were added to fresh extracts (Fig. 2,a), the pattern of recovery was similar to that with freeze-dried tissue (Fig. 2,b) except that the highest percentage recovery obtained with fresh oak roots was only 70% in the TPD extractant. Although the amount added (w/v) was the same, the soluble form of PVP appeared to prevent inhibition of ADH standards by the tannins in fresh oak roots better than insoluble PVPP. Recovery of endogenous ADH from fresh oak roots was also higher in the extractions which contained soluble PVP compared to the insoluble PVPP (Fig. 1). Since the freeze-dried

Table 3. Phenolic and tannin content of water oak and green ash roots (n=3).
*
Values with the same superscript (within row) are not significantly different (P>0.05), based on Duncan's multiple range test.

| Variable | ash | oak |
|---|---|---|
| total phenolics | a 2.39 ± (0.03) | b 7.12 ± (0.02) |
| hydrolyzable tannins | a 0.00 ± (0.00) | b 18.06 ± (0.97) |
| condensed tannins | a 0.00 ± (0.00) | b 2.13 ± (0.15) |

*
Means and standard errors are given for total phenolics and hydrolyzable tannins, expressed on the basis of tannic acid equivalent % of dry roots. Means and standard errors of condensed tannins are expressed on the basis of bisulfited quebracho equivalent % of dry roots.

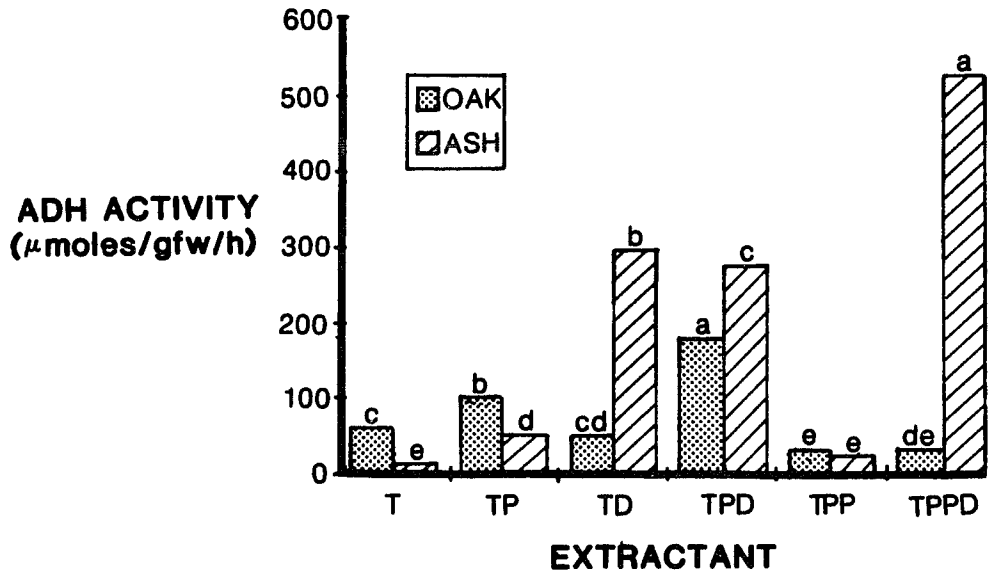


Figure 1. Effect of extractant on alcohol dehydrogenase (ADH) activity (μmoles $g^{-1}$ fr. wt. $h^{-1}$) in water oak and green ash root extracts (n=4). T=100 mM Tris buffer, TP=Tris + 10% soluble polyvinyl - pyrrolidone (PVP), TD=Tris+ 20 mM dithiothreitol (DTT), TPD=Tris + PVP + DTT, TPP=TRIS + insoluble polyvinylpolypyrrolidone (PVPP), TPPD=TRIS + PVPP + DTT. Bars with same letter (within species) are not significantly different ($P>0.05$) based on Duncan's multiple range test.

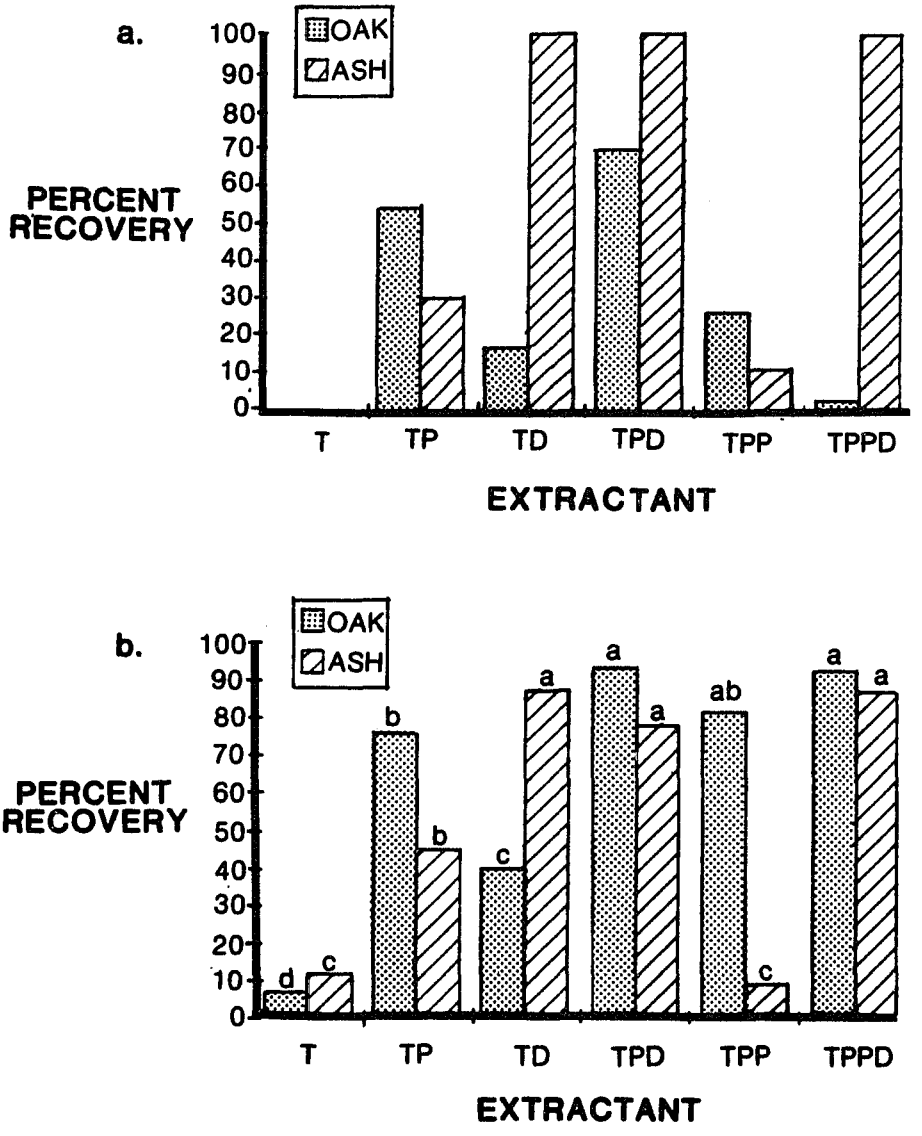


Figure 2. Effect of extractant on alcohol dehydrogenase (ADH) standard recovery (%) from a) fresh (n=1) and b) freeze-dried (n=4) water oak green ash root extracts. See Figure 1 definitions of symbols.

tissue extracted without protective additives caused almost complete inhibition of added standard ADH, it does not seem likely that cryodessication reduced the "tanning capacity" of the oak root tissue (Fig. 2). However, the finely-ground, freeze-dried tissue may have allowed a more immediate interaction of the PVPP with the oak tannins before the enzyme was affected. These results suggested that ADH extraction from freeze-dried roots might be more successful than from fresh tissue.

When enzyme activity measured in fresh roots was compared to that measured in freeze-dried tissue, it was clear that freeze-drying enhanced the extraction and recovery of ADH by 60% from oak roots and 18% from ash roots (Table 4). Loomis (1974) has reported that freeze-dried plant preparations are not suitable for enzyme and organelle isolation since these constituents remain in intimate contact with secondary compounds and in a powder with a large surface area. However, the results of this experiment indicate that cryodessication may, in fact, enhance the extraction and recovery of ADH from tissues with large amounts of phenolic compounds, particularly tannins. It should be emphasized that the roots which were freeze-dried were first rapidly frozen in a layer of ice in liquid nitrogen to ensure that no melt-back of the tissue occurred during handling and transfer to the freeze-dryer. Since the freezing process causes rupture of the plant cells, even minor thawing of the tissue before it is completely lyophilized would allow the interaction of the enzyme with the phenolic substances released from the vacuoles. The use of freeze-dried and finely-ground tissue may

Table 4. Alcohol dehydrogenase activity* ($\mu$moles $g^{-1}$ d wt $h^{-1}$) measured in fresh** and freeze-dried green ash and water oak roots (n=4). Means with the same superscript (within row) do not differ significantly (P>0.05), based on Duncan's multiple range test. Values are mean ± (s.e.).

| species | fresh | freeze-dried |
|---|---|---|
| oak | $755 \pm (35)^{a}$ | $1213 \pm 3^{b}$ |
| ash | $3135 \pm (116)^{a}$ | $3699 \pm 73^{b}$ |

* Root tissue was extracted with 100 mM Tris, pH 7.5, 20 mM DTT, and 6% PVP, or 6% PVPP for oak or ash, respectively.

** Enzyme activity measure in the fresh roots was converted to a dry weight basis: fresh/dry = 3.48 for oak, fresh/dry = 5.50 for ash.

have 1) allowed a more complete extraction of ADH due to increased surface area and 2) allowed a more immediate and intimate interaction of the absorbants, PVP and PVPP, with polyphenolics, thus more effectively preventing enzyme inhibition. Rapid freezing in liquid nitrogen, prevention of tissue melt-back, and storage of the dried tissue in a vacuum dessicator probably contributed to the success this method. Since this experiment was only a preliminary one and conducted primarily to explain the conflicting results observed in the recoveries of ADH from fresh and freeze-dried oak root extracts (Fig. 2, a&B), further investigation of the efect of cryodessication on the interaction of enzymes such as ADH with phenolic compounds is warranted.

The high recoveries of endogenous and standard ADH from ash root extracts when dithiothreitol (DTT) was included in the extraction solution indicated that the active inhibitory phenolic substances in this tree species were primarily quinones (Table 2, Figs. 1 & 2). Quinones, which are highly reactive secondary compounds, are readily oxidized from phenols (Loomis and Battaile, 1966); reducing agents such as DTT, ascorbic acid, and mercaptoethanol prevent their formation. For this reason, the inclusion of DTT in the ash root extractions resulted in high recoveries of ADH (Table 2, Figs. 1 & 2). However, the recovery of endogenous ADH from ash roots was only partially aided by the DTT (Fig. 1) because the phenols themselves react with enzymes. The presence of a polymeric adsorbant was apparently necessary to prevent their interaction with endogenous ADH. PVPP was substantially more effective than PVP in this instance

probably because polyphenolics of lower molecular weight are more readily bound to PVPP (Andersen and Sowers, 1968).

The determination of total soluble protein was also significantly affected by the extractant, particularly with the oak roots (Fig. 3). The oak tannins caused complete precipitation of the soluble proteins in most of the extracts. Only in the TPD extract was there a substantial amount of protein remaining in the supernatant (Fig. 3). The lower molecular weight phenols in the ash roots apparently did not interfere with the protein determination as much as the oak tannins, since measurable levels of protein were detected in all ash extracts (Fig. 3). However, some extractants resulted in significantly more protein than others (Fig. 3). Loomis (1974) reported that the presence of phenolic compounds in plant extracts may result in protein values that are inaccurate by orders of magnitude and recommended that enzyme activities be reported on a "per gram fresh weight" basis. This suggestion seems reasonable in light of the realization that polyphenolic compounds would probably preferentiallly bind to the higher molecular weight proteins, a situation that would result in an overestimation of the activity ocf the enzyme if expressed on a protein basis. This problem would be apparent in tissue extracts in which no protein was detected in the supernatant, but which do have some enzyme activity (oak, Figs. 1 & 3). However, in situations where seemingly reasonable amounts of protein are measured (ash, Fig. 3), the effect of phenolics on protein content might be overlooked. Unless protein standard recoveries are conducted, the effect of phenolic content on protein

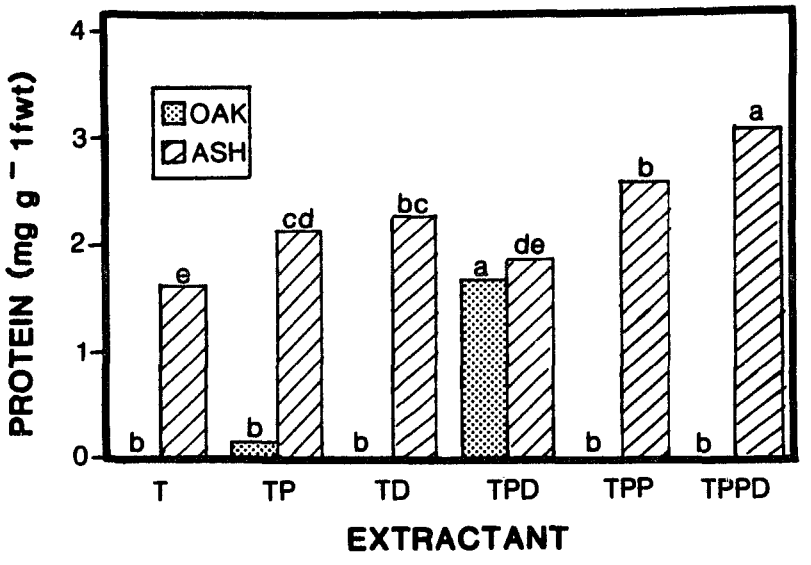


Figure 3. Effect of extractant on root total soluble protein (mg $g^{-1}$ fr wt) in water oak and green ash extracts (n=4). See Figure 1 for definitions of symbols.

determination will be unknown.

Several studies have reported the interference of additives such as mercaptoethanol (Welander, 1978) and PVPP (Salder and Shaw, 1978) with enzyme activity. The concentratiion of PVP and PVPP used had no significant effect on the activity of ADH measured in ash and oak, but did have an effect on protein content in oak (Table 5). Since the effect of protective additives on enzyme activity is generally dependent on the plant species, configuration of the enzyme, and the concentration of the additive; it is advisable to test several types and concentrations with each experimental plant species, particularly if activity is expressed on a protein basis. The optimum pH for the extracton and assay of ADH was 7.5 and 7.0, respectively, for both species (Table 6, Fig. 4). Since the optimum pH for the assay buffer is different from that which has been reported previously for rice ADH (pH 8.0) (John and Greenway, 1976), it is evident that this parameter should be determined individually for each plant species.

In many early studies in which the activity of ADH was measured, extraction of the root tissue was conducted in a buffer with no added protectants (e.g. Keeley, 1979; Francis et al., 1974). In later studies, polymeric adsorbants (PVP and PVPP) and reducing compounds (mercaptoethanol and dithiothreitol) were incorporated into ADH extraction solutions (Wignarajah et al. 1976; Smith and ApRees, 1979a; Mendelssohn et al., 1981; Tripepi and Mitchell, 1984; Jenkin and ApRees, 1983; Campiranon and Koukkari, 1977; Bertani and Brambilla, 1982). Variable recoveries of root ADH due to interference by phenolic compounds could be partially responsible for

Table 5. Effect of PVP or PVPP concentration (% w/v) in the extraction buffer on ADH* activity (μmoles $g^{-1}$ f wt $h^{-1}$) and protein (mg $m^{-1}$ f wt) in oak and ash roots, respectively (n=4). Means with same superscript (within column) do not differ significantly (P>0.05), based on Duncan's multiple range test.

| PVP or PVPP %** | ash | | oak | |
|---|---|---|---|---|
| | ADH | protein | ADH | protein |
| 2 | a 446 ± (32) | a 2.32 ± (0.43) | a 56 ± (9) | a 0.51 ± (0.03) |
| 6 | a 461 ± (19) | a 2.50 ± (0.33) | a 71 ± (9) | ab 0.31 ± (0.13) |
| 10 | a 416 ± (21) | a 1.99 ± (0.31) | a 74 ± (12) | b 0.14 ± (0.08) |

* Roots were extracted in 100 mM Tris buffer, pH 7.5, 20 mM DTT, and the indicated treatment levels of PVPP or PVP, for ash or oak, respectively.

** Values in table are the means of ADH activity ± (s.e.).

Table 6. Effect of extraction buffer pH on ADH activity (μmoles $g^{-1}$ f $wt^{-1}$ $h^{-1}$) and protein content (mg $g^{-1}$ f wt) in ash (n=4) and oak (n=2). Values* with same superscript (within column) do not differ significantly (P>0.05), based on Duncan's multiple range test.

| pH** | ash | | oak | |
|---|---|---|---|---|
| | ADH | protein | ADH | protein |
| 6.0 | b 93 ± (9) | a 1.01 ± (0.19) | a 108 ± (10) | a 0.24 ± (0.17) |
| 7.5 | a 312 ± (14) | ab 1.36 ± (0.13) | a 164 ± (22) | a 0.56 ± (0.22) |
| 9.0 | c 48 ± (4) | b 1.80 ± (0.18) | b 30 ± (0) | a 0.87 ± (0.10) |

* Values in table are the means of ADH activity.

** Roots were extracted in 100 mM Tris buffer, pH 7.5, 20 mM DTT, and the indicated treatment pH levels in PVPP or PVP, for ash or oak, respectively.

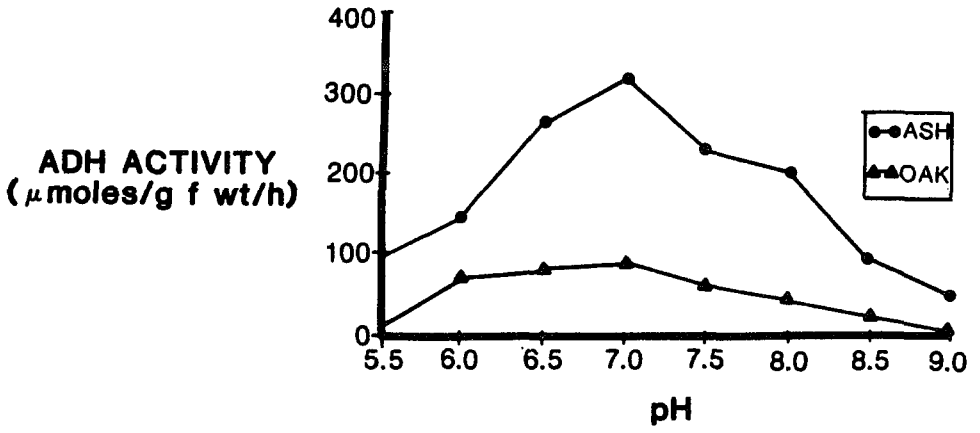


Figure 4. Effect of reaction buffer pH on alcohol dehydrogenase (ADH) activity (μmoles $g^{-1}$ fr wt $h^{-1}$) in water oak and green ash root extracts (n=3). The following buffers were used to achieve the desired pH: 40 mM MES for pH 5.5 and 6.0; 40 mM MOPS for pH 6.5 and 7.0; 40 mM Tris for 7.5, 8.0, and 9.0.

some of the conflicting results reported in the aforementioned studies. If the extraction solution used was not optimized for each plant species, then apparent species and treatment differences in metabolic responses to flooding could have been misinterpreted. In this study, for example, the use of Tris buffer alone or in combination with PVP would have incorrectly shown that during flooding alcoholic fermentation was stimulated to a greater degree in oak roots compared to ash (Fig. 1). Similarly, the use of Tris buffer and insoluble PVPP would have resulted in the conclusion that ADH activity was not stimulated by flooding in either ash or oak roots (Fig. 1). Furthermore, if these results had been expressed on a protein basis, the differential effect of the phenolic compounds on protein determinaton would have contributed significantly to the inaccuracy of both the absolute values and the relative differences between the two species.

The results described in this paper have shown that the use of a reducing agent such as DTT in conjunction with either PVP (in the case of oak) or PVPP (in the case of ash) produced the highest ADH activities and protein values in fresh tree root extracts. The inclusion of these additives in the extraction solutions substantially reduced the interference by the polyphenolic compounds present in the ash and oak roots but did not adversely affect the activity of the enzyme itself. Also, cryodessication of the plant tissue prior to extraction resulted in increased ADH activities measured in oak and ash roots. Because of interest in alcoholic fermentation and other anaerobic pathways in trees and wetland plants

(see Hook and Crawford, 1978; Crawford, 1978; Crawford, 1982; Davies, 1980; Hook, 1984; Kozlowski, 1984), accurate determination of the activity of the enzymes mediating these pathways is essential to the formation of valid theories regarding flood-tolerance. The comparison of enzyme activity among plant species of differing flood tolerance requires the complete extraction of the enzyme from all tissues regardless of phenolic content or treatment. The information presented here may be helpful in achieving this goal.

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# CHAPTER FOUR

# A METHOD FOR CONTROLLING THE WITHIN-ROOT $CO_2$ CONCENTRATION

ABSTRACT. A method is presented for the control of carbon dioxide concentrations within the roots of Fraxinus pennsylvanica Marsh. The results indicated a linear fit of the root $CO_2$ concentrations to the $CO_2$ levels of the treatment gases: $y = 1.1x + 5.4$ ($r = 0.98$, 18 df). The method presented for controlling $CO_2$ can be easily modified for other gas mixtures and plant species.

# INTRODUCTION

The control of the concentrations of gases within roots is of great importance to the study of anaerobiosis. Work in this area has been impeded by the complexity of the phenomenon of flooding. Many factors known to affect root respiration vary concomitantly when water fills the pore spaces of soil. The multiplicity of factors often makes it difficult to interpret the cause-and-effect relationships.

Many researchers have studied the responses of plants to anaerobiosis by flooding them in pots of soil. This method has the advantage of simulating the responses one would expect under field conditions. However, the influences of certain factors remain in question, e.g., the oxygen and carbon dioxide concentrations (Hook et al., 1970); the increased amounts of reduced compounds such as ferrous iron and manganous manganese (Keeley, 1978; Keeley, 1979); and the interactions of soil ethylene, endogenous ethylene production stimulated by flooded conditions (Tang and Kozlowski, 1984), and increased ethylene accumulation within plants due to the jacketing effect of water (Kawase, 1978).

Solution culture is commonly used to circumvent some of these problems. Aerobic vs. anaerobic treatments are often obtained by gassing the solution with either air or nitrogen (e.g., Tripepi and Mitchell, 1984). This approach, however, is somewhat limited. Gases have small diffusion coefficients within aqueous media, and consequently gaseous diffusion within, into, and out of the root is

affected ( Healy and Armstrong, 1972; Saglio, et al., 1984). Another problem is that some gases, such as $CO_2$, will react with dissolved nutrients, and the treatment must be conducted in water rather than in nutrient solution (Geisler, 1963).

Mist chambers have been used with some success to provide a controlled-gas root environment (e.g., Williamson, 1968). I have tried this method with Fraxinus pennsylvanica Marsh.; desiccation symptoms appeared in all of the test plants, and the mortality rate was very high. Also, these systems are somewhat cumbersome to operate, and algae, fungi, and bacteria may cause problems.

The method described in the present chapter is similar to that described by Geisler (1967), but important modifications have been made. The method discussed in this chapter allows for the immediate displacement of soil air and results in a vessel that is gas tight. Geisler had placed plants in perforated containers filled with sand and compost. Five pots were placed into a vessel sealed with a plastic cover with small slits that permitted the tops of the plants to be in air. This method did not allow for immediate displacement of the soil air, nor did it appear to have been gas tight. Geisler did not discuss the results of this method in terms of the internal root gases.

Similar limitations are evident in the method described by Williamson (1970). He used acrylic tubes filled with a sandy loam soil and a layer of loosely packed material at the bottom. Gas entered the cores at the bottom and was eluted through a plastic film covering the soil. One plant was used per core, and gas

consumption was 0.5 liter per minute. I tried this method and found that the resistance of the potting medium to the gas flow results in gas channels along the walls of the containers and along large roots.

The method of controlling root environments elucidated in this chapter allows the researcher to manipulate the $CO_2$ content within roots of green ash (Fraxinus pennsylvanica Marsh.). This methodology should work equally well with other gas mixtures and plant species.

## MATERIALS AND METHODS

The general concept of the use of this chamber is to displace the air with water and then to displace the water with treatment gas. Figure 1 is a diagram of the general construction of the chamber used. The overall dimensions are: length, 122 cm; width, 28.6 cm; height, 30.5 cm. A row of six 4.8-cm-diameter holes that accommodated #10.5 rubber stoppers were drilled along the long axis of the lid. After the holes were drilled, the lid was sawn in half longitudinally. Sixteen bolts were inserted up through the bolt mounts and cemented in place. These were used with wing nuts to affix the lid. All permanent joints were cemented with waterproof epoxy, and the boards were screwed together. The wood was treated with two coats of waterproof sealant and two coats of non-toxic enamel. One additional hole was drilled in the lid and one near the

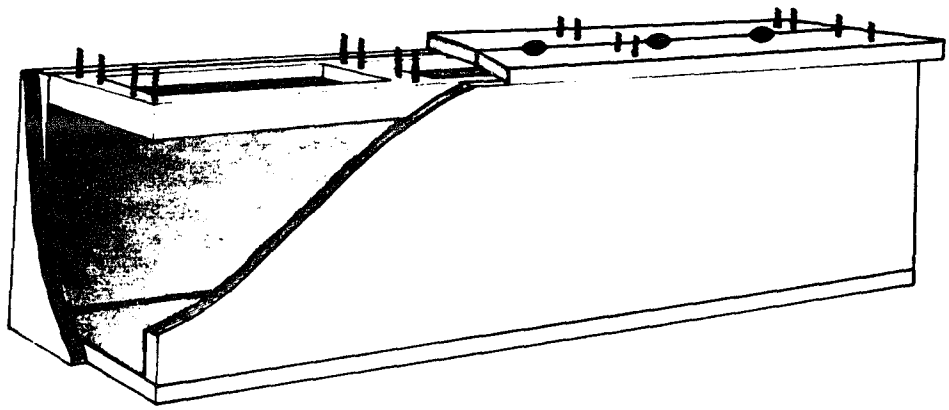

Figure 1. General construction of the chamber. The dimensions are: 122 cm, length; 28.6 cm, width; 30.5 cm, height. Materials shown here are wood and 16 bolts which project upward to allow the lid to be tightened down with wing-nuts.

bottom on the side of the chamber to serve as inlet and outlet ports, respectively. A stopper with glass tubing through it was used at these points to facilitate tygon tubing connections.

A neoprene gasket was cut to fit the plane of the box just below the lid, which helped to secure a gas-tight fit. Nevertheless, leaks occurred around the wing nuts, along the seam down the middle of the lid, and around the trees. Waterproof adhesive tape was subsequently placed along the outside of the box at the top, with an extra 1 cm extending vertically above the box. This allowed for a layer of 3:1 paraffin-petroleum jelly mixture to be poured over the lid (see Plate 1), which eliminated nearly all of the gas leakage. I used a continuous flow-through method, and the decline in gas pressure within the cylinders averaged 1.4 kg $cm^{-2}$ $h^{-1}$.

The trees were grown in a commercial potting mixture of peat and vermiculite, which permitted good drainage and gaseous diffusion. The trees were removed from their pots; the rooting medium was loosened; and the excess (containing no roots) was removed from the root system. The trees were placed in the box, and the top was fitted so that the trunks projected through the holes. Bored, half-split stoppers were placed around the trunks at the root collar and forced into the holes. The chamber was then sealed as described above, and set up in the laboratory under a bank of lights which provided 76 $\mu M$ $m^{-2}$ $s^{-1}$ at mid-foliage level. The lights were set on a 14 h day cycle, and temperature at the foliage level ranged between ca. 21°C (night) and 23°C (day).

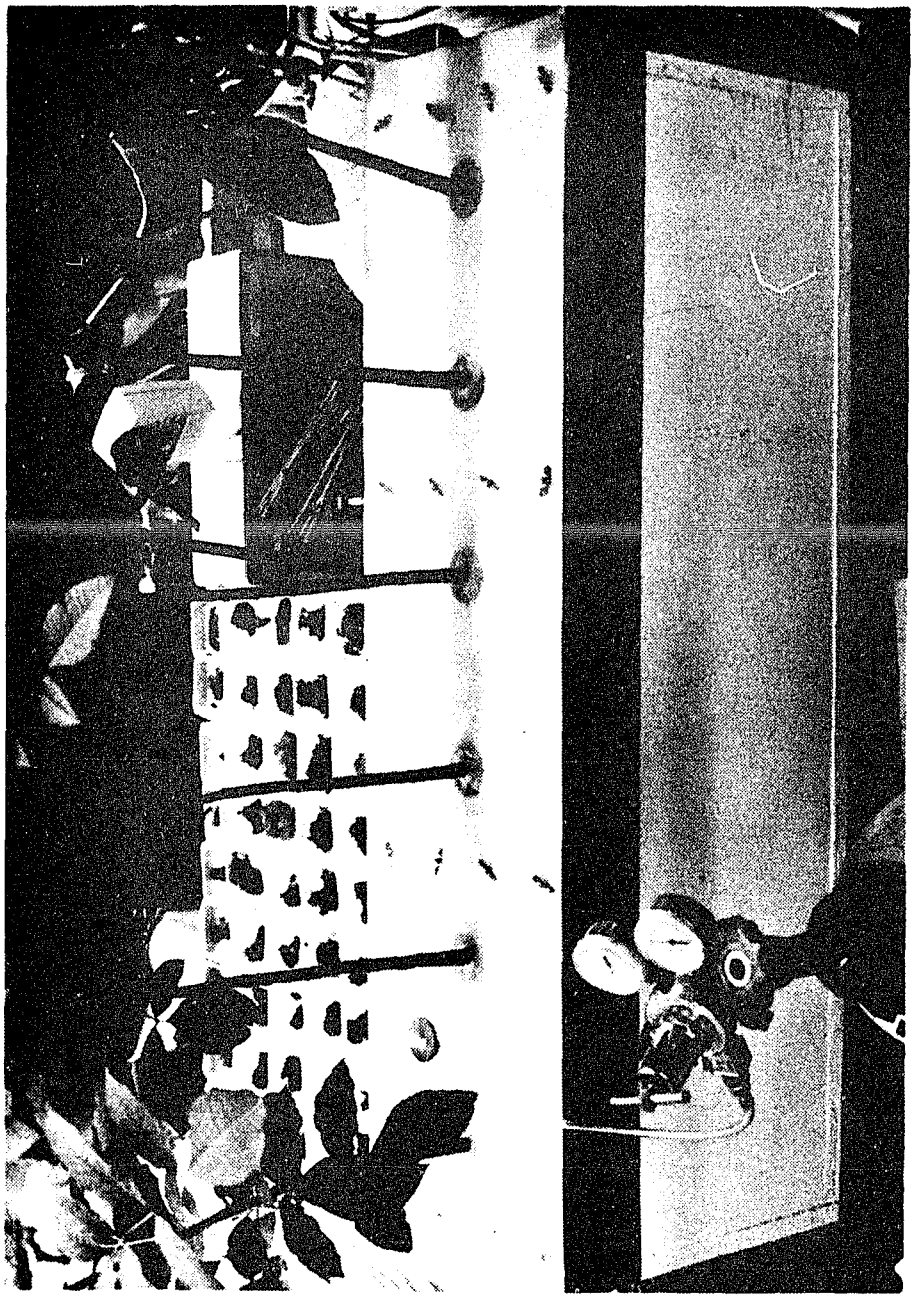

Plate 1. Typical treatment in progress. Tape was placed around the lid so that it extended above the chamber, allowing for a paraffin seal. Tape was also applied along the seam down the middle of the lid, and can be seen through the paraffin. A gas exit port (not shown) was located near the bottom on the side of the chamber. The gas was vented under a slight head of water.

The trees were allowed to acclimatize for three days. The chamber was then filled with water, which displaced the air and also served to water the trees. The box remained filled with water for 30 minutes so that the gases in the rooting medium would be displaced. The treatment gas was then vented into the chamber as the water drained. The treatment gas effluent was bubbled under a slight head of water to prevent back-flow of air. The four treatments used consisted of certified, premixed cylinder gases: 100% nitrogen (0% $CO_2$); and 5% $CO_2$, 21% $CO_2$, and 37% $CO_2$ (balance nitrogen). Each gas was administered for 72 h. Six trees were used as replicates for each treatment, but the total number of observations was 20 because one replicate of each treatment was inadvertently lost.

It should be noted that, if necessary, one could water the root systems by displacing the treatment gas with de-aerated water and then displacing the water with the treatment gas. This was not necessary during these treatments because none of the trees showed any desiccation symptoms, and the rooting medium was still moist at the end of the 72 h treatment.

After completion of the treatments, the trees were removed from the system and immediately placed into an anaerobic glove bag filled with the treatment gas until they could be sampled. The root system was severed from the trunk and placed in a degassed solution of 2M $MgSO_4$ adjusted to pH 2.5 with HCl (Ahn, Collins, and Pharr, 1980). The root gases were collected using the inverted funnel technique described by Beyer and Morgan (1970). A Perkin-Elmer 900B

gas chromatograph equipped with a thermal conductivity detector and an Alltech CTR column was used to measure the $CO_2$ and $O_2$ concentrations. This was a dual column having an inner column containing Porapak P and an outer column containing Molecular Seive. Helium was used as the carrier gas. The mean accuracy of the $CO_2$ concentration determinations was +/- 0.6% (SE=0.2, n=30).

## RESULTS and DISCUSSION

No oxygen was detected in the gas effluent of any of the treatments. The results from the anaerobic treatment indicated a very good linear fit of the resulting root $CO_2$ concentrations to the $CO_2$ levels of the treatment gases: $r = 0.98$, $df=18$. The relationship for predicting root $CO_2$ concentration based on the root environment $CO_2$ was: $y = 1.1x + 5.4$ (Fig. 2).

The levels of internal $CO_2$ measured represented a composite sample from the entire root system: the tap root, secondary and smaller roots. It is likely that the larger roots contributed a disproportionate amount of gas relative to their overall physiological importance. It would be interesting to subsample different regions of the root system, especially the actively growing root tips.

This method of root environment control should have diverse applications. Because of the minimal disturbance to the plants and the possibility of watering them without contaminating the treatment

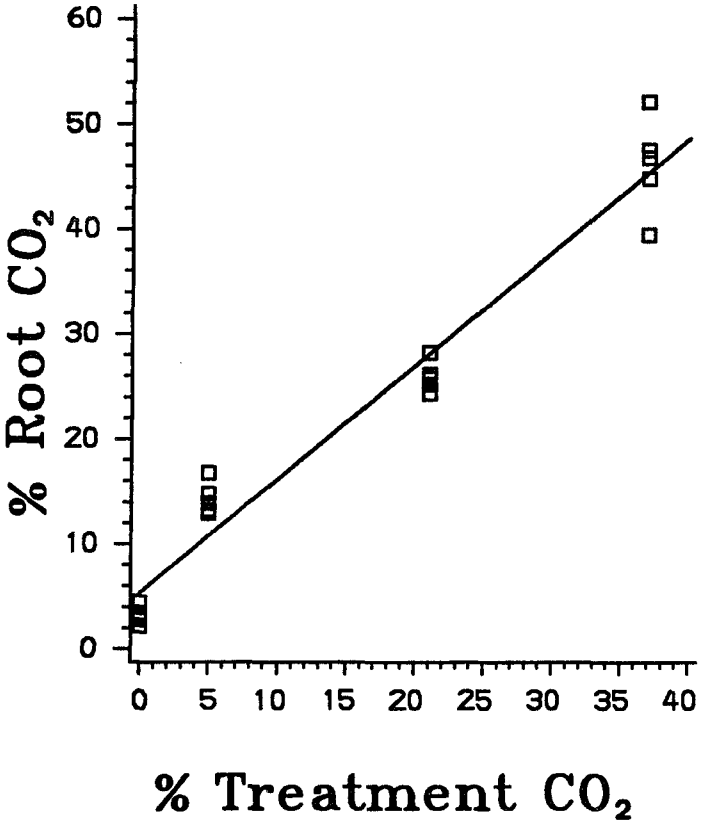


Figure 2. Effect of treatment levels of $CO_2$ on internal root $CO_2$ concentrations: $y = 1.1x + 5.4$ ($r = 0.98$, 18 df). The treatment gases were: 100% nitrogen (0% $CO_2$), 5% $CO_2$, 21% $CO_2$ and 37% $CO_2$ (balance nitrogen). Squares represent individual data points.

gas with air, duration of treatments could conceivably be extended. The data presented here indicate that the level of an internal root gas (in this case, $CO_2$) can be controlled by manipulating the composition of the root environment atmosphere. The method is flexible and could easily be modified to accommodate other plant species and treatments.

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# CHAPTER FIVE

## Effects of Elevated $CO_2$ in Anaerobic Soil Atmospheres on Metabolic Components of Intact Root Systems of Green Ash and Water Oak

ABSTRACT. ADH (alcohol dehydrogenase), ME (NADP-malic enzyme), ethanol, malate, $CO_2$, and $O_2$ within intact root systems of a flood-tolerant tree species, green ash (Fraxinus pennsylvanica Marsh.), were monitored under gaseous anaerobic soil atmospheres which consisted of a range of $CO_2$ concentrations in nitrogen gas. For comparison, an abbreviated series of treatments was also administered to a less flood-tolerant species, water oak (Quercus nigra L.). Linear, statistically significant, increases in ADH and ME activities, and $CO_2$ and ethanol concentrations were found in the green ash roots. These results suggested that $CO_2$ may increase ME and ADH activity by lowering cytoplasmic pH. This would account for some of the observed increases in ADH activity and ethanol production and decreases in malate. Increased soil $CO_2$ resulted in higher levels of ethanol and $CO_2$ in the roots of water oak than in those of green ash. This is discussed relative to the superior flood-tolerance of green ash. The influence of green ash lenticels on gas exchange between roots and above-ground atmosphere was also investigated.

# INTRODUCTION

Although flood-tolerance has been associated with tolerance to elevated levels of soil $CO_2$, the relationship is poorly understood. Hook et al. (1970) found that two swamp tree species, Nyssa sylvativa var. biflora and Nyssa aquatica, responded to elevated levels of soil $CO_2$ with depressed growth. However, these effects could not be dissociated from effects due to lowered concentrations of $O_2$ which were also inversely correlated with $CO_2$ concentrations. In order to eliminate this problem, Hook, Brown and Kormanik (1971) compared the effects of elevated $CO_2$ in a closed liquid culture system on Nyssa sylvativa var. biflora and Liquidambar styraciflua seedlings. They found that the more flood-tolerant species, N. sylvatica var. biflora, withstood elevated levels of $CO_2$ much better than L. styraciflua which died within 15 days in 10% $CO_2$, and 10 days in 31% $CO_2$. Nyssa sylvatica suffered no ill-effects from a 15 d treatment with either 2% or 10% $CO_2$, but at 31%, root development, growth, oxygen uptake, and transpiration rate were retarded.

High partial pressures of $CO_2$ are often present in flooded soils, especially under stagnant conditions. Ponnamperuma et al. (1966) reported that $CO_2$ in flooded soil can at times reach levels of up to 60%, and Hook et al. (1970) measured levels of up to 30%. Therefore, tolerance to elevated soil $CO_2$ may be a common feature among flood-adapted plants. However, the role of soil

$CO_2$ under anaerobic conditions has received relatively little attention since the effects of limiting oxygen concentrations per se are often assumed to be the dominant factor of anaerobiosis (Williamson, 1970; Crawford, 1982). In addition, it is difficult to experimentally control the soil atmosphere while other important parameters are held constant.

The purpose of this study was to describe the response of a flood-tolerant tree to different levels of soil $CO_2$, and to compare these responses with a less tolerant species. I compared selected metabolic responses of green ash (Fraxinus pennsylvanica Marsh.) with those of water oak (Quercus nigra L.), two tree species endemic to bottomland hardwoods of the eastern United States. Green ash is more tolerant of flooding than is water oak and is often found in swamps while water oak is more characteristic of bottomland hardwood communities (Hall et al., 1946; Broadfoot and Williston, 1973). Intact plants with their root systems in controlled soil atmospheres and their aerial organs in air were used. This experimental system maintained the desired compostition of the soil atmosphere surrounding the roots without affecting other variables as is the case when a soil is flooded.

## MATERIALS AND METHODS

The response of root system of Fraxinus pennsylvanica Marsh. to various levels of $CO_2$ in a nitrogen atmosphere were assessed by

monitoring the following variables of the roots: the ADH (alcohol dehydrogenase, EC 1.1.1.1) and ME (NADP-malic enzyme, EC 1.1.1.40) activities, the $O_2$ and $CO_2$ concentrations, and the malate and ethanol concentrations. These responses were compared with those of Quercus nigra L. roots in the treatments specified below.

Plant Material:

One-year old, bare-rooted seedlings of Fraxinus pennsylvanica Marsh. and Quercus nigra L. were provided by the Office of Forestry, La. Dept. of Natural Resources from their nursery at Columbia, La., U.S.A. These were grown as described for green ash in Chapter Three.

Root Atmosphere Control Chamber:

The root atmosphere control chamber used in this experiment is described in Chapter Four.

Experimental Treatments:

Green ash plants were placed in six treatments: 21% $O_2$ in purified nitrogen, 0% $CO_2$ (all $CO_2$ treatment gases were in a balance of nitrogen, and per cent composition is given on a volume basis, exclusive of water vapor); 0% $CO_2$ with silicon, high-vacuum grease applied to the basal 25 cm of the stems; 5% $CO_2$; 21 % $CO_2$; and 37% $CO_2$. Oak seedlings were placed in the following treatments: 21% $O_2$ in purified nitrogen; 0% $CO_2$; and 21% $CO_2$. Six trees were placed in each treatment for 72 hours. After termination of a treatment, the trees were

removed from the system and placed in an anaerobic glove-bag continuously flushed with the treatment gas until samples were collected for internal gases, metabolites, and enzyme activities. Sample collection required about 0.5 h per tree. One treatment was performed per week.

Gas Sampling:

The internal root gases were extracted with the inverted funnel technique of Beyer and Morgan (1970) as described in Chapter Four. The mean accuracies of the $CO_2$ and $O_2$ determinatons were within +/- 0.6% (s.e.=0.2, n=30) and +/- 0.3% (s.e.=0.1, n=26), respectively. Since argon could not be separated from oxygen by this technique, oxygen concentrations included a small percentage of argon, which is present in the atmosphere at 0.93%.

Enzyme and Metabolite Methods:

The grinding buffer used for the extraction of malic enzyme, alcohol dehydrogenase, and protein from the roots consisted of 100 mM Tris HCL; 5-mM $MgCl_2$; 20 mM DTT; 0.5 mM TPP; 6% w/v PVP or PVPP for water oak or green ash, respectively; brought to pH 7.5 with NaOH (see Chapter Three). Small, limber, healthy-looking roots were collected from throughout the root system. These were cut up into approximately 0.5 cm pieces and thoroughly mixed. A 1.0 g sample of tissue was ground in an ice-cold mortar with 10.0 ml of extraction buffer and a small amount of acid-washed sand and then centrifuged for 20 minutes at 15,000 g and $5^{o}C$. One gram of tissue was also

ground in 15 ml of 0.33 N perchloric acid for malate and ethanol determinations using the methods described in Bergmeyer (1974).

The ME (EC 1.1.1.40) assay mixture contained 3.0 ml total volume, 0.5 ml sample extract, 40 mM MOPS (pH 7.0), 2 mM $MgCL_2$, 0.4 mM NADP, and 2.2 mM malate. The reaction was started was started by adding malate to the reaction cuvette. Additional activity due to NADP-malate dehydrogenase, if present, was probably negligible under the conditions of this assay (Johnson and Hatch, 1970).

The ADH (EC 1.1.1.1) assay mixture contained 3.0 ml total volume, 0.1 ml sample extract, 40 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.2 NADH, and 10 mM acetaldehyde. The reaction was started by adding acetaldehyde to the reaction cuvette.

Statistical Analyses:

The data from the six variables; ADH, ME, $O_2$, $CO_2$, malate, and ethanol; were analyzed using preselected contrasts within and between green ash and water oak treatments. The responses of green ash to the following treatments: 0% $CO_2$, 5% $CO_2$, 21% $CO_2$, and 37% $CO_2$, were contrasted based on a set of orthogonal multipliers obtained from the SAS ORPOL procedure (SAS 1982) for the determination of fit for linear, quadratic and cubic models. This was not possible in the case of water oak because of insufficient treatments. See appendix one at the end of this chapter for the means and standard errors of each treatment.

# RESULTS

Alcohol Dehydrogenase and Malic Enzyme:

The ADH activity (Table 1, Fig. 1a) increased significantly in the roots of both species as a result of increased $CO_2$ concentration under anaerobic root conditions. Malic enzyme activity (Table 1, Fig.1b) also increased in both species as a result of increasing $CO_2$ concentrations under anaerobiosis. This relationship was significant as both a linear and quadratic response. In the case of ADH, the quadratic response was the result of an upward inflection between 21% and 37%, while for ME there was a downward inflection at this point.

Although some significant differences in ADH and ME activities between the two species were evident, comparison of enzyme activities between the species was problematical because of unequal recovery rates (see Chapter Three). In a separate experiment, known amounts of ADH and ME were added to the grinding buffers of fresh green ash and water oak roots. The recovery of ADH and ME was 100% and 76% for green ash; and 70% and 29% for water oak, respectively (unreplicated). The difference between species was not surprising, given the extremely high tannin levels characteristic of oaks (see Chapter Three).

Table 1. Probabilities of greater F than observed for selected contrasts.

| Contrast* | ADH | ME | O2 | CO2 | MALATE | ETOH |
|---|---|---|---|---|---|---|
| a) Ash vs Oak: Air | 0.3984 | 0.0412 | 0.0001 | 0.0562 | 0.2080 | 0.0001 |
| b) Ash vs Oak: 0% CO2 | 0.0405 | 0.2321 | 0.2416 | 0.9618 | 0.0034 | 0.0001 |
| c) Ash vs Oak: 21% CO2 | 0.0442 | 0.0451 | 0.2098 | 0.0001 | 0.1907 | 0.0001 |
| d) Ash: Air vs 0% CO2 | 0.0056 | 0.2115 | 0.0001 | 0.3535 | 0.1739 | 0.2113 |
| e) Oak: Air vs 0% CO2 | 0.9570 | 0.7080 | 0.0001 | 0.0032 | 0.0044 | 0.0001 |
| f) Ash: 0% CO2 vs 21% CO2 | 0.0001 | 0.0006 | 0.2166 | 0.0001 | 0.2583 | 0.0700 |
| g) Oak: 0% CO2 vs 21% CO2 | 0.0001 | 0.0068 | 0.3137 | 0.0001 | 0.4627 | 0.0091 |
| h) Ash: 0% CO2 vs grease | 0.5258 | 0.7939 | 0.0295 | 0.1946 | 0.9256 | 0.1176 |
| i) Ash: linear model | 0.0001 | 0.0043 | 0.0238 | 0.0001 | 0.1498 | 0.0067 |
| j) Ash: quadratic model | 0.0106 | 0.0178 | 0.9752 | 0.8318 | 0.5457 | 0.6029 |
| k) Ash: cubic model | 0.1207 | 0.6355 | 0.8052 | 0.0777 | 0.2193 | 0.9487 |

*Air refers to the aerobic treatment: 21% $O_2$ (the balance gas of each treatment was purified $N_2$); 0% CO2 is equivalent to 100% $N_2$. Grease designates that stopcock grease was applied to basal 25 cm of stem.

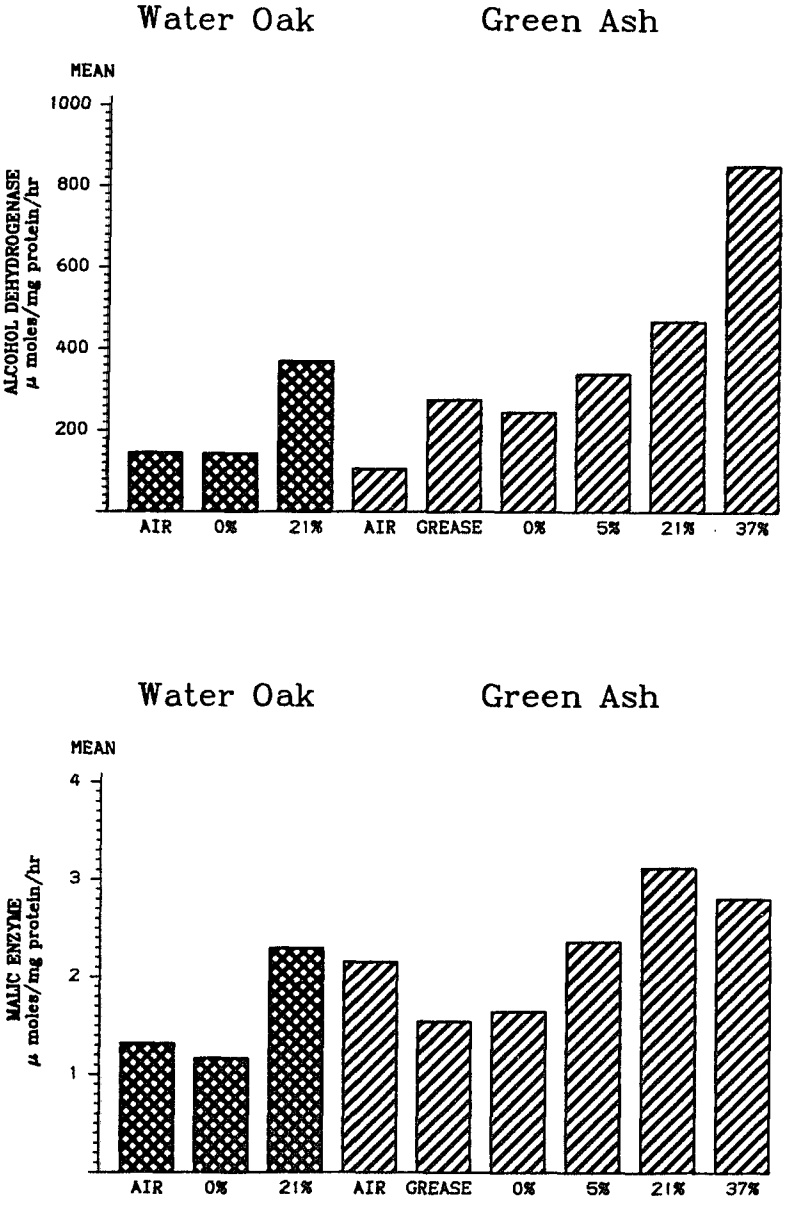


Figure 1. a) Alcohol dehydrogenase activities in water oak and green ash roots. b) NADP-malic enzyme activities. "Air" refers to the aerobic treatment: 21% $O_2$ (balance gas of each treatment was purified $N_2$); 0% $CO_2$ is equivalent to 100% $N_2$; 5%, 21% and 37% refer to treatment levels of $CO_2$ in the soil atmosphere. Grease designates that stopcock grease was applied to basal 25 cm of stem and that the soil atmosphere was 100% $N_2$.

Carbon Dioxide:

Internal $CO_2$ of both species (Table 1, Fig. 2a) was significantly higher in the 21% $CO_2$ treatment than in the 0% $CO_2$ treatment. However, the $CO_2$ concentration within the water oak roots at the 21% $CO_2$ treatment was over twice as high as that of the green ash at this treatment. The internal root $CO_2$ was significantly lower in the water oak aerobic treatment than in 0% $CO_2$. A highly significant linear increase in green ash $CO_2$ with increasing $CO_2$ in the root environment was observed.

Oxygen:

The aerobic treatment of both species resulted in greater internal $O_2$ concentration than the 0% $CO_2$ treatment (Table 1, Fig. 2b). There was significantly higher $O_2$ in the aerobic treatment of green ash than for the aerobic treatment of water oak. In the green ash, the presence of stopcock grease on the basal 25 cm of the stem resulted in a significant decrease in $O_2$ from 4.5% to 2.4%. Also, there was a significant linear decrease in $O_2$ which was associated with increasing concentrations of treatment $CO_2$.

Ethanol:

Ethanol was significantly higher in the oak roots in 0% $CO_2$ than in the aerobic treatment, and in 21% $CO_2$ than in 0% $CO_2$ (Table 1, Fig. 3a). The green ash roots exhibited a significant linear increase in ethanol with increasing $CO_2$. However, there

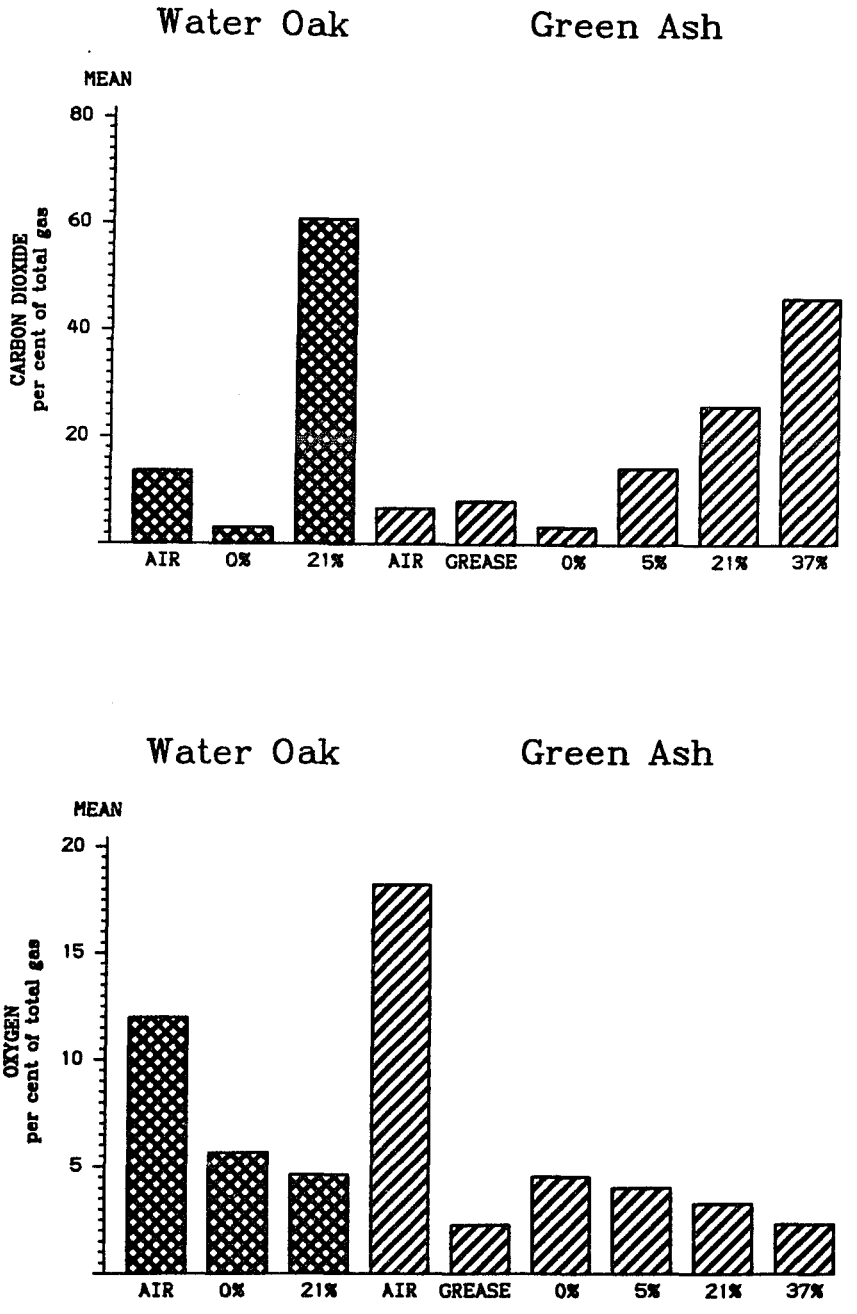


Figure 2. a) Carbon dioxide levels (v/v) in water oak and green ash roots. b) Oxygen levels in water oak and green ash roots. See Figure 1 for explanation of treatments.

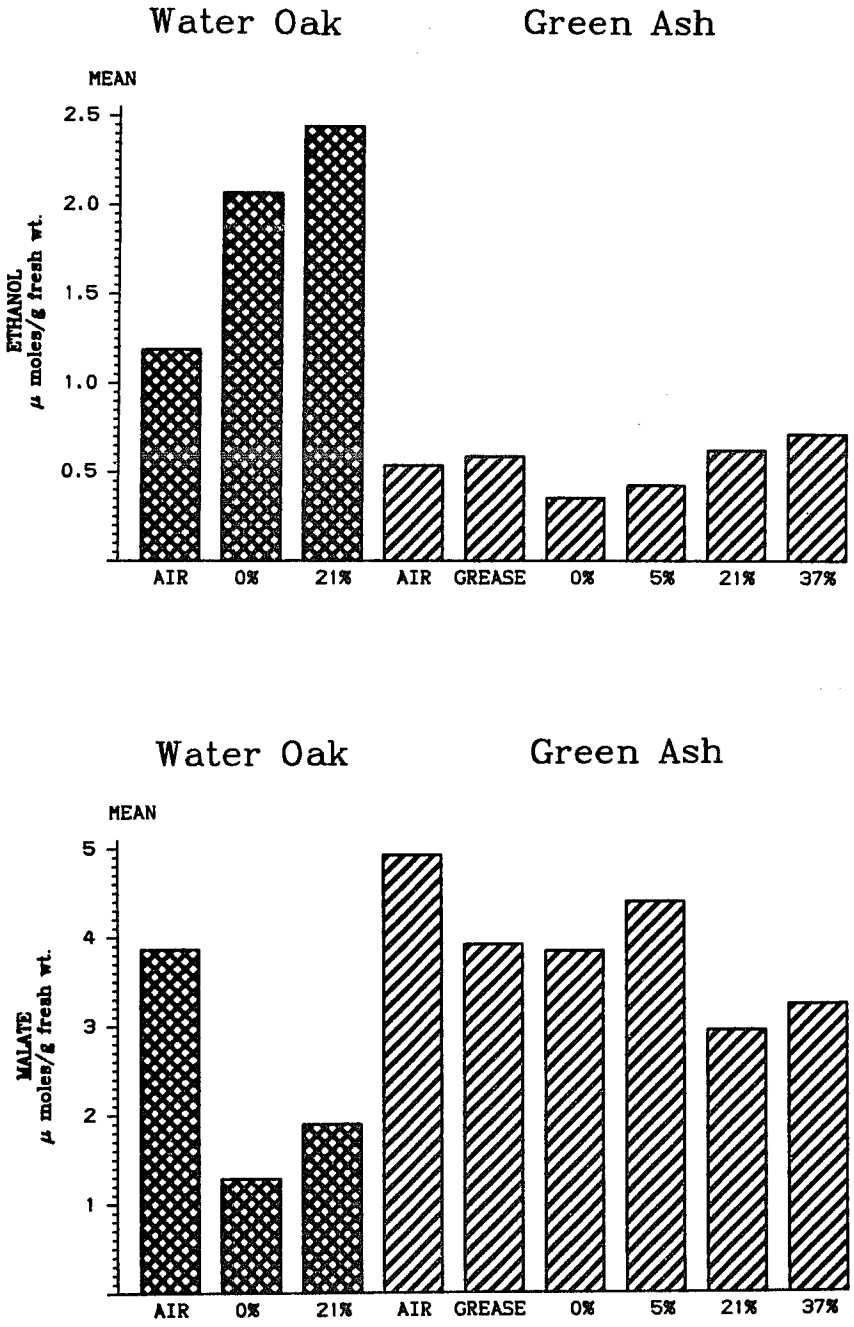


Figure 3. a) Ethanol content of water oak and green ash. b) Malate content of water oak and green ash. See Figure 1 for explanation of treatments.

was no significant difference between the aerobic treatment and 0% $CO_2$ as was the case with the water oak roots. Ethanol concentration in the water oak was consistently greater than for green ash.

Malate:

Malate concentration in the water oak was significantly higher for the aerobic treatment than in 0% $CO_2$ (Table 1, Fig.3b). The only significant interspecific difference among malate concentrations was at the 0% $CO_2$ treatment: green ash contained more.

## DISCUSSION

Both species responded to elevated $CO_2$ under anaerobic conditions with significantly higher ethanol concentrations and ADH and ME activities (Fig.. 1, a & b). This is evidence that elevated $CO_2$ levels affected the rate of anaerobic respiration in both species. Although no previous studies have investigated the effects of $CO_2$ on anaerobic root metabolism of intact plants, enhanced alcoholic fermentation and malate metabolism resulting from elevated $CO_2$ concentrations have been reported to occur in pea seeds (Wager, 1961; Wager, 1974) and detached roots (Chang, et al., 1983; Roberts et al., 1984). In each of the aforementioned cases, the authors suggested that these responses played a role in buffering cytoplasmic pH.

The results of this study suggest that the increasing levels of $CO_2$ lowered the pH of the cytoplasm which in turn was responsible for the increased activity of ME in both species. Davies (1973) pointed out that under physiological concentrations of substrates, ME has an acidic optimum (viz., at pH = 6.6). Increased activity of ME would decarboxylate malate faster and thus provide more pyruvate which, under the conditions of this experiment, was apparently shunted into ethanol production via ADH (Chirkova et al., 1974).

Roberts et al. (1982) demonstrated that hypoxia can reduce the cytoplasmic pH of maize root tips and they suggested that alcoholic fermentation prevented a further decline in pH through the associated formation and release of $CO_2$ from the cytoplasm. An addition of exogenous $CO_2$ under hypoxic conditions further reduced the cytoplasmic pH, and this additional pH drop reduced the length of time that maize root tips tolerated hypoxia (Roberts et al., 1984). Maize genotypes deficient in active ADH likewise responded to hypoxia with accelerated cytoplasmic acidosis and diminished survival (Roberts et al., 1984). Their results indicated that the superior flood tolerance of maize in comparison with the flood-sensitive pea was related to the former's greater ability to prevent cytoplasmic acidosis. Similarly, the increase in alcoholic fermentation in green ash may help buffer cytoplasmic pH when $CO_2$ concentrations are elevated during hypoxia.

Correlations between ethanol accumulation and flood intolerance have been reported by many workers. For example, Barta (1984) compared flood-intolerant Medicago sativa L. with flood-tolerant

Lotus corniculatus L.: the flood-intolerant species accumulated ethanol to a much greater extent. Likewise, Crawford and Baines (1977) found that under flooded conditions flood-tolerant Pinus contortus accumulated only 0.7 umoles $g^{-1}$ fresh wt. of ethanol as compared to 5 umoles $g^{-1}$ fresh wt for flood-intolerant Picea sitchensis under similar conditions. The assumption that these levels are toxic has been disputed (Jackson et al., 1982); however, this issue has not been resolved because differences in exogenously and endogenously produced ethanol has not been adequately addressed to date.

The metabolic switch hypothesis states that flood-tolerant plants avoid alcoholic fermentation by stimulating alternate anaerobic pathways which produce less toxic products such as malate (Crawford, 1978); i.e., flood-tolerant plants would have greater anaerobic/aerobic malate levels than flood-intolerant plants. In this experiment neither green ash nor water oak accumulated malate under anaerobiosis. However, there was significantly more malate in the oak roots under aerobic conditions as compared with the 0% $CO_2$ treatment, whereas green ash malate at 21% $O_2$ and 0% $CO_2$ were not significantly different (Fig.3b, Table1). Thus, the anaerobic/aerobic malate levels in green ash (78.0%) and water oak (33.2%) correspond to those suggested by the metabolic switch hypothesis.

There is evidence from several different species that malate decarboxylation under anaerobic conditions may be more characteristic of flood-intolerant than flood-tolerant plant species. Tripepi and

Mitchell (1984) reported a similar decline in malate concentration in the roots of two tree species, Betula nigra L. and Betula pendula Roth., under anaerobic solution culture. In their work, as in the present study (Fig. 3b), anaerobobic conditions stimulated a much greater decline in malate in the less flood-tolerant species. Similarly, in a study involving roots from three flood-tolerant species, Smith and ap Rees (1979a) reported a very small decrease in malate concentration resulting from 240 min anaerobiosis (0.20 to 0.91 umole $g^{-1}$ f wt); however, in roots of flood-intolerant Pisum sativa, the anaerobic treatment induced a decrease in malate of 2.29 umoles $g^{-1}$ fr. wt (Smith and ap Rees, 1979b).

Differences in aeration and metabolic responses are not mutually exclusive, and it is likely that both contributed to the observed differences between green ash and water oak in response to elevated soil $CO_2$. Water oak roots consistently had higher levels of ethanol than green ash, and responded to elevated $CO_2$ levels with much higher internal $CO_2$ levels. These two facts may be partly responsible for the superior flood tolerance of green ash, and both could have resulted from differences in root aeration. Better aeration of the green ash roots was indirectly indicated by the fact that they contained about 6% more $O_2$ than the water oak roots under aerobic conditions (Fig. 2b). However, the fact that the water oak roots contained over twice as much $CO_2$ under the 21% $CO_2$ treatment than the green ash roots (Fig. 2a), suggests that differences in respiration may also be partly responsible for the much higher levels of $CO_2$ in the oak roots.

When the basal 25 cm of green ash were blocked with stopcock grease, there was a significant decrease in root $O_2$ (Table 1). This response was less dramatic than expected (Armstrong 1968). It is likely that flood-acclimatized seedlings would have demonstrated a much greater dependence on lenticels for aeration. After about two weeks of continuous flooding, green ash seedlings produce hypertrophied lenticels, and adventitious roots after four weeks; whereas water oaks do not (see Chapter Six). One would expect that after the induction of these traits which enhance root aeration (Hook et al., 1971; Sena Gomes and Kozlowski, 1980; Tang and Kozlowski, 1984), the differences in ethanol and $CO_2$ accumulation (see Chapter Six) would be more pronounced than in the non-flood-acclimated seedlings used in this experiment.

Flood tolerance in plants is usually ascribed either to morphological or physiological processes. Several investigators contend that flood tolerance is dependent on the ability to avoid anaerobic conditions by possessing a well developed air-space system which allows for the movement of air through the plant to roots (Webb and Armstrong, 1983; ap Rees and Wilson, 1984). Contrary to this, other researchers have indicated that physiological adaptations are of paramount importance: e.g. the metabolic switch hypothesis, wherein the flood tolerants accumulate relatively non-toxic malate in lieu of ethanol (McManmon and Crawford, 1971; Linhart and Baker, 1973); and the compensatory hypothesis whereby flood tolerance results from ATP production arising from increased alcoholic fermentation (Hochachka and Somero, 1973; Tripepi and Mitchell,

1984). However, the ecological importance of elevated soil $CO_2$ concentrations (Hook et al., 1970; Ponnamperuma et al., 1966) has been virtually ignored. This study indicated that, contrary to prior assertions (Williamson, 1970; Crawford, 1982), soil $CO_2$ can result in significant physiological responses that are probably mediated in part by differences in morphology, and that these responses are relevant to flood tolerance.

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Appendix 1. Means and standard errors of ADH, ME, O2, CO2, MALATE, and ETOH for all treatments.*

| Trt. | ADH:mean (s.e.),n | ME | O2 | CO2 | MALATE | ETOH |
|---|---|---|---|---|---|---|
| (ASH) 21% O2 | 104.19 (8.58), 6 | 2.15 (0.11), 6 | 18.2 (0.4), 5 | 6.7 (0.9), 5 | 4.91 (0.70), 6 | 0.54 (0.04), 6 |
| 0% CO2+ grease | 274.28 (40.40), 6 | 1.54 (0.26), 6 | 2.3 (0.4), 5 | 8.1 (0.7), 5 | 3.91 (0.76), 6 | 0.58 (0.06), 6 |
| 0% CO2 | 243.62 (10.50), 6 | 1.65 (0.26), 6 | 4.5 (0.3), 5 | 3.2 (0.4), 5 | 3.83 (0.75), 6 | 0.35 (0.05), 5 |
| 5% CO2 | 337.62 (18.20), 6 | 2.36 (0.16), 6 | 4.0 (1.0), 5 | 14.3 (0.7), 5 | 4.38 (0.47), 6 | 0.42 (0.04), 6 |
| 21% CO2 | 467.17 (21.94), 6 | 3.11 (0.19), 6 | 3.3 (0.3), 5 | 26.0 (0.6), 5 | 2.93 (0.41), 6 | 0.62 (0.06 |
| 37% CO2 | 850.02 (68.46), 6 | 2.81 (0.37), 6 | 2.3 (0.2), 5 | 46.2 (2.1), 5 | 3.22 (0.46), 6 | 0.71 (0.11), 6 |
| (OAK) air | 145.07 (14.02), 6 | 1.32 (0.45), 6 | 12.0 (0.7), 6 | 13.6 (0.8), 6 | 3.86 (0.71), 5 | 1.19 (0.12), 6 |
| N2/ 0% CO2 | 142.47 (9.71), 6 | 1.17 (0.16), 6 | 5.7 (1.1), 6 | 3.0 (0.4), 6 | 1.28 (0.19), 5 | 2.07 (0.12), 6 |
| 21% CO2 | 367.92 (52.35), 6 | 2.30 (0.38), 6 | 4.6 (0.8), 4 | 61.1 (7.5), 5 | 1.89 (0.25), 6 | 2.45 (0.17), 6 |

*Air refers to the aerobic treatment: 21% $O_2$ (the balance gas of each treatment was purified $N_2$); 0% CO2 is equivalent to 100% $N_2$. Grease designates that stopcock grease was applied to basal 25 cm of stem.

# CHAPTER SIX

## Gas Composition and Respiration of Water Oak and Green Ash Roots After Prolonged Flooding

ABSTRACT. The effects of a 9.5 months continuous flooding treatment was compared with a drained control treatment on one-year-old seedlings of green ash (Fraxinus pennsylvanica Marsh.) and water oak (Quercus nigra L.), two tree species common to the bottomland-hardwood forests of eastern North America. The internal root gas composition of the more flood-tolerant species, green ash, maintained higher oxygen and lower carbon dioxide concentrations under the flooding treatment than water oak. This apparently resulted in differences in rhizosphere oxidation. The amounts of Fe and Mn and the Fe/Mn ratio of the root coating extracted from trees in reduced soil conditions were much higher for the green ash than the water oak. It is argued that this reflects differences in the ability of these two species to maintain rhizosphere oxidation under prolonged periods of flooding and to prevent the accumulation of reduced and potentially phytotoxic compounds. Alcohol dehydrogenase activity increased in the green ash and decreased in the water oak in the flooded treatment. This indicated that the better adapted species was able to rely upon increased anaerobic respiration in order to compensate for the decreased root oxygen supply, but the water oak was unable to maintain previous levels of respiration, probably as the result of sulfide toxicity.

# INTRODUCTION

The varying degrees of flood tolerance exhibited by tree species results in the well known correlations of forest type with flooding intensity-duration gradients (Day and Monk, 1974; Bell and DelMoral, 1977; Peet and Loucks, 1977; Keeley, 1979; McEvoy et al., 1980). Green ash (Fraxinus pennsylvanica Marsh.) is considered to be a flood-tolerant tree (Hook and Brown, 1973), while water oak (Quercus nigra L.) is less so (Patrick et al., 1981). This suggests that the root systems of these two species will respond differently under conditions of prolonged flooding, and that a comparison of these differences should shed light on the underlying adaptive mechanisms.

The permeability of roots to gases from the aerial part of the stem can be an important adaptation to flooding because this provides for greater aerobic respiration and for oxidation of the rhizosphere. Aerobic respiration is much more efficient than anaerobic: 36 moles of ATP per mole of glucose vs. two, respectively. Rhizosphere oxidation can ameliorate the effects of several potential phytotoxins characteristic of reduced soils, e.g. reduced iron and manganese, and $H_2S$. Thus, the diffusion of gases from the atmosphere into the roots is likely to be an important characteristic of the relatively few tree species able to endure prolonged periods of flooding. The purpose of this study was to investigate the response of a tree species which is adapted to prolonged perions of flooding, green ash, and compare this with a less flood tolerant tree species, water oak.

The variables examined included the internal root gas composition; the accumulation of Fe, Mn, and P in the leaf tissue and root coating; and the root alcohol dehydrogenase activity, malate and soluble protein.

## MATERIALS AND METHODS

### Plant Material:

One-year-old, bare-rooted seedlings of green ash and water oak were obtained from the Office of Forestry, La. Dept. of Natural Resources from their nursery at Columbia, La.

### Soil:

A Commerce silt loam was obtained from the plow layer of a field at the St. Gabriel Sugar Cane Experiment Station, La. This soil had previously been under sugar cane cultivation. An average of 5,000 g was added to two-gallon plastic pots which had drainage holes in the bottom.

### Experimental Design:

The treatments were applied in a 2 x 2 factorial arrangement. Either a green ash or water oak seedling was placed in each pot. All trees were grown under well-drained conditions prior to the flooding treatment. Flooding consisted of submerging the pots in a tank of water so that the soil surface was at a depth of 10 cm. The

treatment and control groups were kept under a shelter made of transparent polyethylene sheeting material which permitted 71% transmission of ambient sunlight intensity. The sides of the structure were open.

All control pots were watered whenever any of the trees showed signs of wilting. The flooding treatment began in mid-July and continued until the beginning of May, the following year - approximately 9.5 months. The water level in the flooding pots was maintained throughout the entire period. The trees were dormant from mid-March until late December.

Soil Redox Potential:

Redox potential (Eh) measurements were taken in duplicate in order to assess the variation within each pot. Platinum-tipped electrodes were inserted to a depth of 6 cm and allowed to equilibrate overnight before reading against a calomel reference electrode. The control pots were assayed one day after they were watered.

Growth:

Trees were weighed before potting and then again upon harvest. It was assumed that growth prior to flooding was relatively uniform and did not affect the differences between species and treatments.

ADH, Malate, Protein, and Root Coating Analyses:

The buffer used for the extraction of ADH (alcohol dehydrogenase, EC 1.1.1.1) and soluble protein from the roots consisted of 100 mM

Tris HCL; 5 mM MgC12; 20 mM TPP; 6% w/v PVP or PVPP for water oak or green ash, respectively; brought to pH 7.5 with NaOH (see Chapter Three). Two samples were ground per tree for ADH and protein analyses. Small, limber, healthy roots were collected from throughout the root system. These were cut up into about one-cm pieces and thoroughly mixed. A 0.5-g sample of tissue was ground with a mortar and pestle in 5.0 ml of extraction buffer and then centrifuged for 20 minutes at 15,000 g and $5^{o}C$. The ADH assay mixture contained 3.0 ml total volume, 0.1 ml sample extract, 40 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.2 NADH, and 10 mM acetaldehyde. The reaction was started by adding acetaldehyde to the reaction cuvette.

One gram of tissue was also ground in 5 ml of 0.33 N perchloric acid for malate determination using the method described in Bergmeyer (Bergmeyer, 1981).

One gram of tissue was also taken from the collection of roots for root coating analysis as described by Mendelssohn and Postek (1982). See Chapter Seven for details of the method. The extract was analyzed with an ICAP (Inductively Coupled Argon Plasma) spectrometer.

Analyses of Soil and Root Gases:

A plexiglass tube, 1.5-inch outside diameter, was used to collect soil samples. A plunger, a rubber stopper attached to a wooden dowell, was used to create a suction which aided sample extraction as the tube was inserted into the soil. A head of water was maintained above the plunger to help prevent the back-flow of air into the space

above the sample. The sample core included soil down to the bottom of the pot. The samples were extruded into a solution of 2M $MgSO_4$ adjusted to pH 2.5 with HCL. The gases were collected under vacuum using the inverted funnel technique of Beyer and Morgan (1970).

The internal root gases were also extracted with the inverted funnel technique. Immediately after samples were taken for the root coating, ADH, protein, and malate assays, the root systyem was severed from the stem. This sample consisted of the tap root, secondary roots and the remaining smaller roots, and it was placed in a $MgSO_4$ solution as described above. The gases were collected and analyzed before the next tree was removed from its pot. Although the sampling was done as quickly as possible, some gas exchange with air was unavoidable during this process.

Two gas samples were collected for each soil and root observation: one for ethylene and one for $O_2$ and $CO_2$. A flame ionization detector equipped with a Porapak-N 80/100 mesh column was used for ethylene measurements. For $CO_2$ and $O_2$, a thermal conductivity detector with an Alltech CTR column was used (see Chapter Three).

Leaf Elements:

All of the leaves from each tree were dried at 75°C for 48 hours. The tissue was ground and a one-gram sample was redried to constant weight, and digested in 25 ml of "Baker Instra-analyzed" nitric acid. The digestion procedure was as follows: the samples

were left over night at room temperature then gradually brought up to 85°C over a two-hour period, then heated at 85°C for five hours. The digestions were diluted to 100 ml using glass distilled water and analyzed using an ICAP spectrometer.

## Results and Discussion

The low Eh values of the flooded soils (Table 1) indicated that most of the inorganic redox systems were in their reduced states. The Eh values of the drained pots were measured one day after watering and therefore represent lower than average values; nevertheless, they indicated that nearly all of the major inorganic redox couples were in their oxidized states (Gambrell and Patrick, 1978).

The soil $CO_2$ concentrations in the flooded treatments were high, especially for the pots with green ash (Table 1). After 9.5 months of continuous flooding, one would expect that the total amount of gas would be small; therefore, the high percentages of $CO_2$ did not necessarily represent very much total $CO_2$, but total volumes were not measured. Also, some $CO_2$ could have come out of solution during the extraction process due to the low pressures I used and the low pH of the extraction solution. The statistically significant interaction of the soil $CO_2$ for the treatment-by-species interaction effect (Table 1) was evidently an expression of the large differences between species in the flooded

Table 1. Soil gases and Eh

a) Means and (standard errors)

| Treatment | Species | $CO_2$ % | $O_2$ % | Ethylene ppm | Eh mv |
|---|---|---|---|---|---|
| drained | Ash | 0.8 (0.1) | 20.7 (0.1) | 0.28 (0.09) | 163 (34) |
| drained | Oak | 1.8 (0.6) | 20.6 (0.7) | 0.12 (0.02) | 208 (33) |
| flooded | Ash | 55.6 (2.3) | 2.0 (0.4) | 0.60 (0.04) | -202 (48) |
| flooded | Oak | 38.0 (3.8) | 1.2 (0.2) | 0.56 (0.07) | -223 (40) |

b) F values and significance levels of above variables

| Source | $CO_2$ | $O_2$ | Ethylene | Eh |
|---|---|---|---|---|
| treatment | 414.39** | 2032.31** | 39.83** | 101.49** |
| species | 13.73** | 1.12 NS | 2.76 NS | 0.75 NS |
| trt×sp | 17.39** | 0.72 NS | 1.13 NS | 0.40 NS |
| subsamples | — | — | — | 0.89 NS |
| observations | 20 | 20 | 20 | 40 |

**, p>F = 0.01; NS, p>F is non-significant.

treatment. The higher soil $CO_2$ concentration associated with the green ash probably resulted directly from root respiration or from respiration by soil organisms which were exploiting root exudates, and indicated greater overall metabolic activity by green ash roots than water oak. The soil ethylene was higher in the flooded treatment than in the undrained, as would be expected (Smith and Restall, 1971).

The root gas data (Table 2) indicated that there was more $CO_2$ and ethylene and less $O_2$ in the roots of both tree species subjected to the flooded treatment. The $CO_2$ and ethylene concentrations were higher and the $O_2$ was lower in the water oak roots of both treatments than in green ash. Hook and Brown (1972) demonstrated that the vascular cambium of green ash is permeable enough to permit aeration of the living cells of the root xylem with air which entered the tree at the lenticels of the lower stem (Armstrong, 1968; Hook et al., 1971). I observed hypertrophied lenticels and basal swelling of the green ash from one to two weeks of after flooding and adventitious roots after four weeks, whereas the water oak seedlings exhibited only slight enlargement of basal lenticels and no discernable swelling after 9.5 months of flooding and produced no adventitious roots. These differences would account for the ability of green ash to maintain higher $O_2$ and lower $CO_2$ levels under flooded conditions (Table 2). Comparable differences likely explain this same trend in the drained treatment. The high level of ethylene in the flooded oak may be a symptom of physiological stress (Yang and Pratt, 1978).

Table 2. Root gases

a) Means and (standard errors)

| Treatment | Species | $CO_2$ % | $O_2$ % | Ethylene ppm |
|---|---|---|---|---|
| drained | Ash | 2.4 (0.4) | 20.0 (0.4) | 0.30 (0.13) |
| drained | Oak | 9.3 (1.2) | 10.1 (0.9) | 1.01 (0.16) |
| flooded | Ash | 10.4 (3.6) | 14.1 (1.2) | 0.56 (0.00) |
| flooded | Oak | 15.0 (0.6) | 8.0 (1.0) | 2.78 (0.34) |

b) F values and significance levels of above variables

| Source | $CO_2$ | $O_2$ | Ethylene |
|---|---|---|---|
| treatment | 12.28** | 18.84** | 23.43** |
| species | 8.80** | 73.53** | 49.01** |
| trt×sp | 0.33 NS | 4.11 NS | 12.95** |
| observations | 20 | 20 | 20 |

**, p>F = 0.01; NS, p>F is non-significant.

Root aeration differences have been pointed out as a major factor directly related to differences in flood tolerance and inversely related to drought tolerance in several species (Hook and Brown, 1972; Keeley, 1979). I have likewise found that water oak is more drought tolerant than green ash (unpublished data).

There was a significant increase in the root coating concentrations of Fe and Mn (Table 3) for both species as a result of flooding. Although species differences were not statistically significant, the green ash increased Fe ten times more than water oak while the differences in Mn were not as great.

The Fe/Mn ratio should be a sensitive indicator of the oxidation status of root surfaces in reduced conditions: (1) Mn oxidation takes place at a much slower rate than iron, (2) the critical redox potential of iron oxidation is lower and will occur before Mn oxidation, (3) there are much greater amounts of reduced iron at the low redox potentials represented by this flooding treatment (Patrick, 1980; Patrick and Henderson, 1981), and (4) because the ratio cancels differences in absolute amounts between observations. A comparison of this ratio in the flooded green ash and water oak supports this contention (Table 3), especially in light of the differences in root oxygen concentration (Table 2). However, the decrease in this ratio for the water oak roots upon flooding cannot be attributed to oxidation-reduction reactions alone. It is possible that preferential oxidation or absorption of iron by mycorrhizae accounts for the relatively high value of this ratio in the drained treatment (Bowen, 1973), and that the relatively high amount of Mn on the roots

Table 3. Root coating Fe, Mn, Fe/Mn and P

a) Means and (standard errors)

| Treatment | Species | Fe mg/g.fw | Mn mg/g.fw | Fe/Mn | P mg/g.fw |
|---|---|---|---|---|---|
| drained | Ash | 0.058 (.009) | 0.009 (.002) | 6.65 (1.06) | 0.167 (.027) |
| drained | Oak | 0.281 (.033) | 0.016 (.002) | 18.30 (1.41) | 0.166 (.012) |
| flooded | Ash | 3.64 (.006) | 0.22 (.014) | 17.97 (0.31) | 0.618 (.190) |
| flooded | Oak | 1.78 (.161) | 0.25 (.046) | 6.99 (2.88) | 0.188 (.023) |

b) F values and significance levels of above variables

| Source | Fe | Mn | Fe/Mn | P |
|---|---|---|---|---|
| treatment | 16.73** | 30.94** | 0.00 NS | 5.33* |
| species | 1.74 NS | 0.30 NS | 0.02 NS | 4.40 NS |
| trt×sp | 2.82 NS | 0.15 NS | 28.41** | 4.38 NS |
| observations | 19 | 19 | 19 | 19 |

**, p>F = 0.01; ×, p>F = 0.05; NS, p>F is non-significant.

of water oak in the flooded treatment is due to absorption and coprecipitation with feric oxyhydroxide rather than oxidation of the Mn per se (Krauskopf, 1972). Phosphorus apparently coprecipitated with the iron under the flooded treatment onto the green ash roots (Table 3) as a result of sorption onto ferric oxyhydroxide (Patrick and Khalid, 1974; Chen et al., 1980).

The Fe/Mn ratios in the digested leaves (Table 4) indicated a significant species-by-treatment interaction. It is apparent that this was due to an increase in the iron content of the oak leaves. This increase did not show up as a significant effect in the iron data per se, probably due to the large variability in the data; but the mean of the iron in the oak leaves is over four times as large as its drained treatment counterpart. The trends in this ratio for the leaf data (Table 4) were the reverse of those for the root data (Table 3). This supports the hypothesis that uptake of reduced compounds is inversely related to the oxidation capacity of the root (Bartlett, 1961).

Work done with Spartina alterniflora Loisel. would indicate that in areas where it is unable to oxidize its rhizosphere, $H_2S$ is the main toxic agent. Spartina alterniflora "die-back" occurs in those areas under natural conditions that allow $H_2S$ production (Mendelssohn et al., 1981). However, in artificial systems with equivalent amounts of reduced Fe and Mn, but without $H_2S$ accumulation, its growth was not affected (DeLaune et al., 1984). Spartina alterniflora growing under extremely reduced conditions is unable to precipitate iron and manganese on its root surfaces to the

Table 4. Leaf Fe, Mn, Fe/Mn and P

a) Means and (standard errors)

| Treatment | Species | Fe mg/g.dw | Mn mg/g.dw | Fe/Mn | P mg/g.dw |
|---|---|---|---|---|---|
| drained | Ash | 0.102 (.005) | 0.026 (.022) | 4.11 (0.38) | 3.22 (.261) |
| drained | Oak | 0.078 (.003) | 0.087 (.016) | 1.06 (0.26) | 1.13 (.052) |
| flooded | Ash | 0.086 (.006) | 0.067 (.014) | 1.47 (0.31) | 1.38 (.182) |
| flooded | Oak | 0.337 (.161) | 0.133 (.046) | 5.16 (2.88) | 1.80 (.077) |

b) F values and significance levels of above variables

| Source | Fe | Mn | Fe/Mn | P |
|---|---|---|---|---|
| treatment | 2.27 NS | 3.06 NS | 0.25 NS | 12.56** |
| species | 1.98 NS | 6.38* | 0.05 NS | 25.26** |
| trt×sp | 2.90 NS | 0.01 NS | 5.30* | 57.02** |
| observations | 20 | 20 | 20 | 20 |

**, p>F = 0.01; ×, p>F = 0.05; NS, p>F is non-significant.

same extent as S. alterniflora growing under less severe reducing conditions (Mendelssohn and Postek, 1982). If, under sufficiently reduced conditions, the roots are able to precipitate relatively high amounts of iron (as compared to Mn) then we can assume that $H_2S$ has likewise been oxidized to nontoxic sulfate. When I ground the flooded oak roots for enzyme, protein and metabolite analyses, I noticed the distinctive smell of $H_2S$ - this smell was absent from all other treatments. This indicated that the oxidative capacity of the flooded oak roots was exceeded, and this in turn resulted in the buffering of the rhizosphere Eh by the sulfate-sulfide redox system. Therefore, the roots were unable to prevent hydrogen sulfide from entering the root system, and only small amounts of reduced iron and manganese were removed at the root surface. The large amounts of Fe and Mn in the leaves (Table 4), and the small Fe/Mn ratio of the root coating data (Table 3) corroborated this.

The ADH, malate, and soluble protein of the water oak were all extremely low in the flooded oak (Table 5), probably due to the overall toxic effect of the reduced compounds. There was a four-fold increase in ADH in the green ash roots due to flooding (Table 5) - there was some increased reliance upon fermentation due to the decline in root $O_2$ concentration (Table 2). The data from green ash indicated that malate levels (Table 5) did not increase relative to the drained treatment after 9.5 months of continuous flooding. The decline in green ash protein may be symptomatic of flooding stress on the root system (Table 5). However, the depressed growth of the drained green ash as compared to the flooded may indicate a

Table 5. Tree growth; and root ADH, protein and malate

a) Means and (standard errors)

| Treatment | Species | Growth g | ADH µmol/g.fw/hr | Protein mg./g.fw | Malate µmol/gfw |
|---|---|---|---|---|---|
| drained | Ash | 103.8 (11.0) | 112.3 (6.8) | 1.19 (.07) | 8.61 (0.51) |
| drained | Oak | 76.4 (17.7) | 64.5 (8.2) | 0.57 (.06) | 3.83 (0.91) |
| flooded | Ash | 182.4 (33.3) | 394.3 (75.3) | 0.45 (.10) | 7.18 (0.25) |
| flooded | Oak | 24.2 (14.7) | 20.1 (3.9) | 0.00 (0.0) | 0.20 (0.08) |

b) F values and significance levels of above variables

| Source | Growth | ADH | Protein | Malate |
|---|---|---|---|---|
| treatment | 0.40 NS | 8.65** | 84.33** | 22.06** |
| species | 19.59** | 27.32** | 57.24** | 119.42** |
| trt×sp | 9.73** | 16.34** | 1.40 NS | 4.19 NS |
| subsamples | — | 0.01 NS | 0.20 NS | — |
| observations | 20 | 40 | 40 | 20 |

**, p>F = 0.01; NS, p>F is non-significant.

stress of the drained treatment due to insufficient water for optimal growth (Table 5). This suggests that in the case of the green ash in this experiment, the flooding stress impinged directly upon root metabolism due to toxic effects whereas drought stress was integrated throughout the entire plant.

Green ash roots were able to oxidize their rhizospheres under very reduced conditions for 9.5 months. This ability was associated with better exchange of $O_2$ and $CO_2$ in the roots than was observed in water oak under similar conditions (Table 2). However, this did not preclude the need for increased reliance upon anaerobic respiration in the green ash (Table 5). Rhizosphere oxidation was indicated by high levels of Fe and Mn and a high Fe/Mn ratio in the root coating data (Table 3), and apparently prevented excessive accumulation of reduced compounds such as ferrous iron, manganous manganese (Table 4), and hydrogen sulfide by the plant. This study suggests that root aeration and anaerobic respiration contribute to the survival of green ash under periods of continuous flooding of up to 9.5 months.

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# CHAPTER SEVEN

# A Preliminary Evaluation of Root Responses of Green Ash as a Soil-Wetness Indicator

**ABSTRACT.** Green ash (Fraxinus pennsylvanica Marsh.) seedlings were transplanted in plots along four BLH (bottomland hardwood) transects in Louisiana which represented a wide range of soil-moisture, flooding regimes. Each plot was classified as "wet" or "mesic" based on soil wetness data. The following root-coating constituents; Al, As, Ca, Fe, K, Mn, Ni, P and Zn; and the enzyme ADH (alcohol dehydrogenase) of the seedlings were assayed after 1.5 years. A two-group discriminant analysis function was developed in order to determine how well the seedling root data would predict the predetermined site-wetness category of each seedling's plot. The deposition of root-coating constituents and anaerobic respiration were distinct enough from the seedlings of the wet and mesic sites to be useful in the development of a fairly reliable model for site-wetness classification. The percentages of seedlings correctly grouped into the mesic and wet categories were 88.9 and 92.0, respectively. The variables chosen for inclusion in the discriminant analysis function, in descending order of predictive power, were K, Fe/Mn, Ni, Mg, ADH, and Mn.

# INTRODUCTION

The soil conditions associated with waterlogging can have dramatic effects on terrestrial vegetation. At the community level, waterlogged conditions are characterized by flood-tolerant species (Robertson, et al. 1978; Hook 1984). At the individual plant level, these conditions stimulate the expression of adaptations in thoses plant species that are considered to be flood tolerant (Hook et al., 1971; Pereira and Kozlowski, 1977; Sena Gomes and Kozlowski, 1980). The question posed by this investigation is: are the flood-induced responses of a flood-tolerant species of predictive value in determining the wetness of a site?

The unique properties and ecological importance of wetlands have stimulated keen interest in the development of reliable and efficient indicators of soil wetness. Because of the long recognized relationships between floristic composition of a site and soil wetness, there has been a lot of effort directed towards the development of methods to delineate wetlands based on plant communities. The importance of this trend is underscored by the strong emphasis that the present legal definition of "wetlands", as stated in section 404 of the Clean Water Act, places on vegetation:

> "Those areas that are inundated or saturated by surface or groundwater at a frequency and duration sufficient to support, and that under normal circumstances do

> support, a prevalance of vegetation typically adapted to life in saturated soil conditions (Federal Register, p.37128, 1977)."

At the community level, floristic composition and indicator species are two vegetative aspects have been used to some degree to predict wetland conditions. The indicator species approach has met with critism (Gosselink et al., 1981), and has not received much attention. Community composition is the main criterion used by most agencies responsible for wetlands regulation (Alcock et al., 1984). However, standardized techniques have not yet been developed that can account for community differences due to geography, climate, age and disturbance of stands. This creates a potential for significant discrepencies among delineations by different groups. This problem was evident recently when three federal agencies were asked to estimate the portion of the total bottomland hardwood (BLH) area to be within section 404 jurisdiction (i.e. wetlands): Department of the Interior, 86%; Environmental Production Agency, 68%; U. S. Army Corps of Engineers, 60% (Alcock et al., 1984).

An alternate approach would be to look at the expression of plant adaptations to flooding at the individual plant level. However, this has apparently not been utilized for delineating wetlands to date. The objective of this investigation was to measure selected characteristics thought to be directly related to adaptations to waterlogged soil conditions, and to test how reliably they can predict site "wetness". As most of the current controversy over

wetland deliniation focuses on BLH's, a bottomland hardwood tree species, green ash (Fraxinus pennsylvanica March.), was chosen as the study organism.

In the literature relevant to plant adaptations to flooded conditions, two main responses are frequently suggested as adaptive: 1) anatomical and morphological characteristics that increase the oxidizing capacity of the roots (Armstrong, 1972; Roberts et al., 1984), and 2) metabolic adaptations which compensate for inadequate aeration (Rumpho and Kennedy, 1981; Tripepi and Mitchel, 1984).

Within woody species, the ability to aerate root systems with oxygen supplied from emergent organs through lenticels, aerenchymatous tissue, and adventitious roots is of adaptive importance under flooded conditons (Armstrong, 1968; Hook and Scholtens, 1978). Flooding often induces these features in flood-tolerant species (Hook et al., 1971; Pereira and Kozlowski, 1977, Sena Gomes and Kozlowski, 1980). Not only is aeration thought to be important because of the need for effective oxygen supply, but Chirkova and Gutman (1972) have demonstrated that potentially phytoxic metabolic products associated with flooding; ethanol, acetaldehyde, and ethylene; diffuse out of lenticels.

Since several inorganic compounds are soluble in their reduced form but insoluble in their oxidized form (e.g. Fe and Mn), they tend to precipitate on the root surface as a consequence of an oxidizing root within a reduced soil environment. This process forms an oxidized coating or plaque on the root surface, the amount of which is generally thought to form as a function of the amount of reduced

Fe and Mn present (Bacha and Hossner, 1977; Taylor et al., 1984) and the oxidizing capacity of the roots (Bartlett, 1961; Mendelssohn and Postek, 1982).

"Anaerobic respiration" refers to the plant metabolic responses that occur as a result of oxygen depletion. Probably the most intensively studied step of this metabolic response is the increased activity of the enzyme alcohol dehydrogenase. This enzyme (EC 1.1.1.1.) catalyses a step of the fermentative process which yields energy from the breakdown of sugar in the absence of oxygen. The by-products of this reaction include ethanol and $CO_2$. As ethanol is thought to be potentially phytotoxic (McManmon and Crawford, 1971; Barta, 1984), there has been an on-going debate as to whether a flood-induced stimulation in ADH is representative of an adaptive or a maladaptive response to flooding (Crawford and Bains, 1977; Davies, 1980; Jackson et al., 1982). However, green ash is apparently unusual in that the ratio of ethanol accumulation under anoxic conditions versus aerobic conditions is quite high, even among flood-acclimated individuals (Hook and Brown, 1973; Hook and Scholtens, 1978). Therefore, one would expect that ADH would increase in this species under flooded conditions.

Based on the above considerations, root coating constituents were chosen as an indicator of rhizosphere oxidation activity, and alcohol dehydrogenase activity was selected as an expression of anaerobic respiration. The hypothesis to be tested by this investigation was: anaerobic respiration and rhizosphere oxidation are indicative of waterlogged soil conditions.

## MATERIALS AND METHODS

### Study Sites:

Four study areas were chosen in alluvial bottomlands with mature, BLH overstory which represented a broad range of the available parent material types in Louisiana (Fig. 1). The "Pearl River" transect was located on the Pearl River Wildlife Management Area, St. Tammany Parish, and is underlain by Pearl River alluvium. The "Red River Bay" transect is located on the Grassy Lake Wildlife Management Area, Avoyelles Parish, and is underlain by Red River alluvium. The Spring Bayou transect, located on the Spring Bayou Wildlife Management Area, Avoyelles Parish, is underlain by Mississippi River alluvium. The "Quimby" transect was located on private land near Quimby, Madison Parish, and is also underlain by Missippi River alluvium.

Within each area, a transect was established along the major topographic elevational gradient (Table 1). Three or four plots were subjectively chosen along each transect so as to represent the main soil-moisture, flooding regimes and community types available. There were native green ash (Fraxinus pennsylvanica Marsh.) present on each of the transects (Table 1).

### Plant Material:

One-year old, bare-rooted seedlings of green ash were obtained from the Office of Forestry, Louisiana Department of Natural Resources from their nursery at Columbia, La., U.S.A.

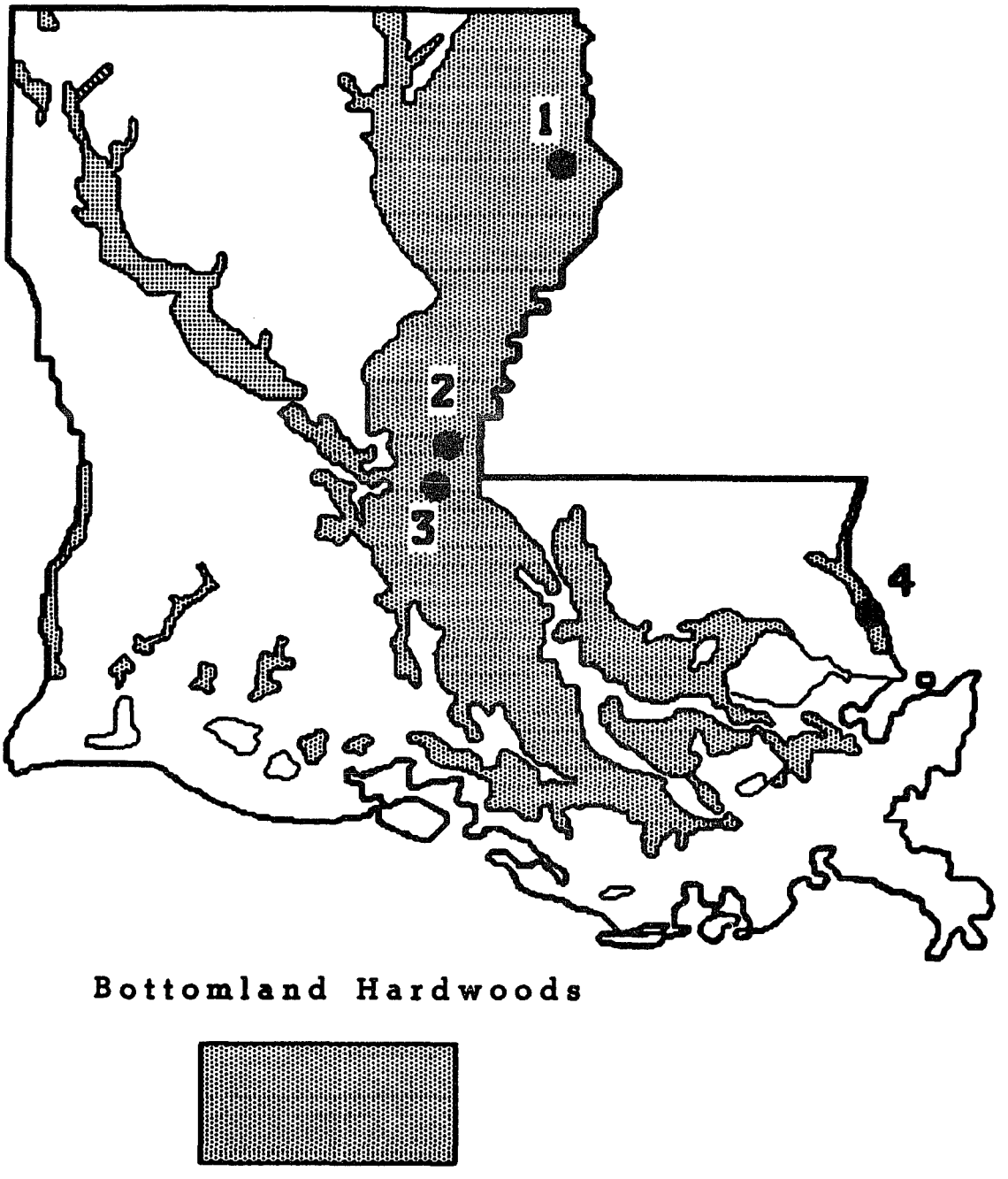


Figure 1. Locations of study transects within Louisiana. The Quimby transect was located at "1", Red River Bay at "2", Spring Bayou at "3", and Pearl River at "4".

TABLE 1. Site characteristics of study transects.*

| transect/ plot | Soil Series | pH of A horizon | % base sat of A | I.V.** overstory green ash | I.V.** understory green ash | relative elevations (meters) |
|---|---|---|---|---|---|---|
| Pearl River | | | | | | |
| one | Arkabulta | 3.9 | 20.0 | - | - | 0 |
| two | Rosebloom | 4.1 | 24.4 | - | - | -0.67 |
| three | Rosebloom | 4.2 | 31.7 | 9.9 | 4.4 | -1.34 |
| four | Rosebloom | 4.5 | 29.7 | 5.7 | - | -1.52 |
| Red River Bay | | | | | | |
| one | Norwood | 7.2 | 82.5 | 12.2 | 33.9 | 0 |
| two | Norwood | 7.6 | 80.9 | - | 37.4 | -1.43 |
| three | Moreland | 7.2 | 81.9 | - | - | -3.72 |
| Spring Bayou | | | | | | |
| one | Tensas | 4.5 | 58.1 | - | 19.8 | 0 |
| two | Tensas | 5.5 | 77.8 | - | 42.9 | -0.30 |
| three | Kobel | 5.0 | 67.7 | 3.3 | 11.2 | -1.04 |
| Quimby | | | | | | |
| one | Goldman | 4.8 | 48.4 | - | - | 0 |
| two | Kobel | 5.6 | 68.7 | - | - | -1.04 |
| three | Kobel | 4.5 | 65.5 | 3.5 | 34.3 | -1.86 |
| four | Fausse | 5.6 | 62.2 | 102.1 | 58.3 | -2.62 |

* See Figure 1 for transect locations.

** I.V. (importance values) based on summation of relative density and relative dominance (for overstory), and relative frequency (for understory).

Plant Response Analytical Methods:

Thirty to 35 one-year-old, bare-rooted seedlings were planted during the late dormant period (March) of 1982. Root samples from four seedlings per plot, were taken from late July - early August, 1983, and then again in September 1983. The roots from each tree were assayed to determine alcohol dehydrogenase (ADH) activity and root coating constituents.

ADH Extraction and Assay:

The grinding buffer used for the extraction of ADH from the roots consisted of: 100-mM Trizma HCl (adjusted to pH 7.3 with Trizma base); 5-mM $MgCl_2$; 0.5-mM TPP; 20-mM DTT; 10% w/v PVP (Anderson, 1968; John and Greenway, 1976). Approximately 0.5 g of roots were removed from a seedling, washed, rinsed in distilled $H_2O$, and placed into a small preweighed plastic bag with 5.0 ml of grinding buffer in the field. This was then immediately frozen on dry ice. Upon return from the field, bags containing the frozen samples were weighed for root-weight determination. The samples were allowed to thaw in ice-cold mortars, ground, and analyzed for ADH within two hours. The sample extracts were assayed at $30^{o}C$ in the following reaction mixture: 2.8 ml total volume, 5.4 mM $MgCl_2$, 0.26 mM NADH, 0.40 mM acetaldehyde, in 14 mM Tris buffer at pH 8.0 (John and Greenway, 1976).

Root Coating Analysis:

A separate sample of root tissue was collected from each seedling for the determination of root coating compostition using a modification of the method of Jackson (1958). This basic method has been utilized by Bacha and Hossner (1977), Mendelssohn and Postek (1982) and Taylor et al. (1984). The samples were rinsed of excess soil with distilled water, patted dry, placed in plastic bags and immediately frozen on dry ice in the field. Upon return to the laboratory, these samples were kept frozen until analyzed. After about one month, the samples (ca. 0.5 g fr. wt) were weighed, and placed in a beaker containing 40 ml of 0.3 M Na-citrate and 5 ml of 1.0 M Na-bicarbonate at 80$^{o}$C. One g Na-dithionite was added to this while the solution was stirred. The suspension was kept at 80$^{o}$C for 15 min. After determination of volume, the solution was filtered through 45 um filters and stored with 0.04 g EDTA per sample. Two blanks were run for every batch of ten samples and the average level of blank elements was subtracted from the samples of that batch. The extract was analyzed with an ICAP (Inductively Coupled Argon Plasma) spectrophotometer for the following elements: Al, As, Ca, Fe, K, Mn, Ni, P, and Zn.

Soil data:

Soil oxidation-reduction potential (redox), oxygen status, and water content data were collected at monthly intervals from duplicate sets of permanently installed equipment at each plot at four depths: 15, 30, 60, and 120 cm. The sampling equipment was only accessable

when the depth of surface water was less than about 15 cm. Thus, unavoidably, more data was available throughout the year from the higher plots. Water-table depth was measured in two perforated PVC pipes in gravel-lined wells to a depth of 120 cm at each plot.

a) water content:

I used a Troxler model 3222 depth moisture gauge (a neutron probe device) using aluminum access tubes to measure the water content (weight per volume) of the soil.

b) $O_2$:

The soil oxygen content was estimated using soil-atmosphere equilibrium chambers similar to that described by Patrick (1977). Inverted platic centifuge tubes; vol = 30 $cm^3$, diameter = 2.8 cm, length = 6.5 cm; were buried to the desired depth and the contents were accessed via a 0.3 cm o.d. copper tubing which had a three-way valve at the aerial end. A sample of the gas from within the chamber was removed using a syringe and measured with a Yellow Springs model 51B oxygen meter. This gas was then reinjected into the chamber.

c) redox potential:

Soil redox potential (Eh) data were collected using permanently installed, mercury-filled, bright platinum electrodes. The redox values were read using an Orion model 231 millivolt meter and a calomel reference electrode.

Statistical Methods:

a) establishment of "wetness" gradient:

A two-step approach was taken in order to group the plots as

either "wet" or "mesic" on the basis of the soil wetness data. Since a large number of soil-moisture variables were collected, and all of them above were highly correlated with each other ($P < 0.001$ for each pair-wise comparison), prinicpal component analysis was used as the first step so that the number of variables and the problems due to multicollinearity could be reduced. This method produces a set of principal components, which maintains most of the information about the variation in the original data. The principal components were calculated using the SAS (1982) "Princomp" procedure after first standardizing the data (mean = 0.0, variance = 1.0). The data were collected during 1983 and consisted of the mean from each pair of replicate measurements from each plot on the four transects studied.

The second step was to classify the plots into either of two groups, wet or mesic, based on the means of the first and second principal components. Principal components are not intercorrelated and are therefore useful in cluster analysis. The plots were grouped based on the first two clusters produced by the SAS "Cluster" procedure. The algorithm specified was Ward's method (SAS, 1982). It produces an agglomerative, hierarchical set of clusters.

A two-stage approach was also used in the development of the model to predict plot wetness from the root data. The initial step was to choose the best predictors from among the root variables. All possible predictor variables (ADH, Al, As, Ca, Fe, Fe/Mn, K, Mg, Mn, Ni, P, and Zn) were entered into the SAS "Stepdisc" procedure, along with the plot wetness classification for each sampled tree. This

method selected the variables which contributed most to the discriminatory power of the model. The options chosen for the procedure (SAS, 1982) were the stepwise discriminant selection method (Srivastava and Carter, 1983), and an entrance criterion of $P > F = 0.15$ (Costanza and Afifi, 1979).

Following the selection of variables for use in the discriminant model for the combined transect data, the "Discrim" procedure (SAS, 1982) was used to develope and characterize a model to predict the plot wetness group (wet or mesic) from standardized root variable data. The probability of misclassification of each model was determined by two techniques: 1) the *a posteriori* resubstitution method (Lachenbruch and Mickey, 1968; Green 1978, p.176), in which the samples used to construct the discriminant function are then used to determine its misclassification rate; and the jackknife method (Tukey, 1949; Lachenbruch and Mickey, 1968; Sokal and Rohlf, 1981), wherein each observation is left out in the development of the model and then used in turn to test it.

SEM Examination:

Roots of green ash seedlings were collected from plots one and three of the Spring Bayou transect during April of 1983. The seedlings were two years old at this time, and had been growing on the transect for a year. Several of the healthiest terminal root segments were chosen from one seedling at each plot. These were rinsed in deionized water, and stored in 2% GTA fixative in 0.02 M phosphate buffer, pH 7.2 in the field. Several days later they were

washed in buffer, then in deionized water, and dehydrated in a graduated ethanol series followed by critical point drying (Postek et al., 1980). They were coated with approximately 200 A gold-palladium, and photographed using a Hitachi S-500 SEM at 25 KeV.

## RESULTS

### Principal Components Analysis:

The first two principle components ("prin1" and "prin2") accounted for 75.5% of the variance in the 13 soil variables, and were the only ones that had eigenvalues greater than one (Table 2). The third principal component only accounted for an additional 7.6%. I therefore decided to retain only the first two for further analysis. An analysis of the factor loading indicated that all of the original variables loaded fairly uniformly on prin1, while water content data appeared to dominate prin2 (Table 2). Water content and water depth were inversely related to the other variables of prin1, which suggested that this axis reflected the original intercorrelations of the soil data in response to waterlogging. At the high values of prin1 (the mesic end), soil moisture and water depth were low, while the redox potential and oxygen levels were high, the opposite was true at low values of prin1. A high value for prin2 would indicate a relatively high soil moisture content. The plots are shown relative to their average prin1 and prin2 values in Figure 2. A varimax rotation of factor scores was performed in order to facilitate

Table 2. Principal components analysis of standardized (m=0,s=1) soil variables from all 14 plots (124 observations).

| variable** | EIGENVECTORS* prin. 1 | prin. 2 | $h^2$ |
|---|---|---|---|
| WC15 | -0.269 | 0.388 | 0.223 |
| WC30 | -0.241 | 0.477 | 0.286 |
| WC60 | -0.251 | 0.456 | 0.271 |
| WC120 | -0.244 | 0.385 | 0.208 |
| EH15 | 0.281 | 0.208 | 0.122 |
| EH30 | 0.298 | 0.180 | 0.121 |
| EH60 | 0.292 | 0.126 | 0.101 |
| EH120 | 0.271 | 0.063 | 0.077 |
| OX15 | 0.283 | 0.198 | 0.119 |
| OX30 | 0.308 | 0.239 | 0.152 |
| OX60 | 0.294 | 0.189 | 0.122 |
| OX120 | 0.262 | 0.144 | 0.089 |
| WDEP | -0.301 | -0.130 | 0.107 |
| eigenvalues | 7.8607 | 1.9607 | |
| cum. % | 60.47 | 75.55 | |

* $h^2$ is the communality: the variance accounted for by the first two principal components.

** abbreviations for variables are as follows, WC15 is the water content (weight/vol) at 15 cm, etc.; EH is the redox potential; OX is oxygen content; and WDEP is the water-table depth.

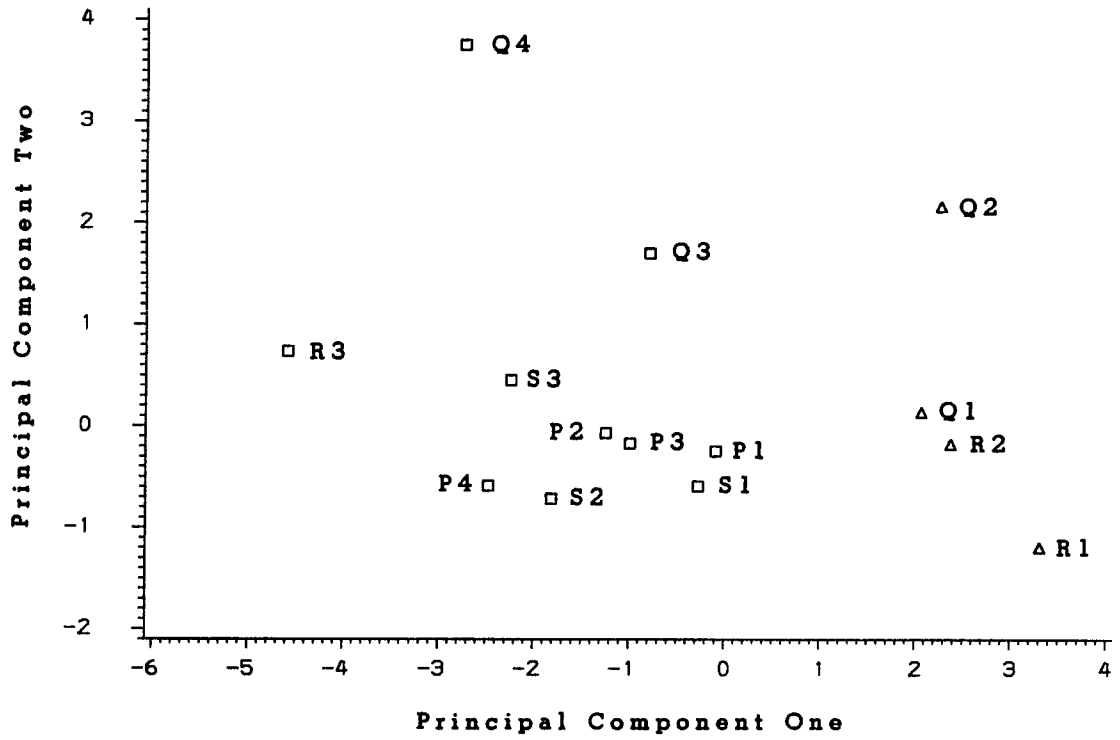


Figure 2. Mean of principal component one and two for transect soil wetness data. The letters "P", "R", "S", and "Q" designate Pearl River, Red River Bay, Spring Bayou, and Quimby, respectively. Numbers refer to plot number. The square symbols are the "wet" plots, triangles are "mesic".

interpretaton of the principal components. This resulted in a shift of the factor loadings of the water content from prin1 to prin2. As this did not affect the above interpretation, the results of the varimax rotation are not presented.

Cluster Analysis:

The group memberships based on the "Cluster" procedure are indicated on Figure 2. The procedure was rerun using the average linkage algorithim instead of Ward's, and the membership of the two largest clusters was identical. The majority (10) of the plots were classified as "wet", and only four; plots one and two at Quimby and Red River; were grouped into the "mesic" category.

Discriminant Analysis:

A wet/mesic classification variable was added to the data set of each seedling, based upon the plot it had come from. The stepwise discriminant analysis then identified which of the root variables were "best" able to determine whether the seedling had come from a wet or a mesic plot. These variables, in descending order of their partial correlation coefficients, were: K, Fe/Mn, Ni, Mg, ADH, and Mn (Table 3).

The above variables were used in the discriminant analysis. First, the null hypothesis of homogeneity of within covariance matrices (Table 4) of the two wetness groups was tested. The test is a likelyhood ratio test (Kendall and Stuart, 1968). The resulting chi-square test value was 201.20 (21 df): P > chi-square = 0.0001.

Table 3. Summary of forward stepwise discriminant analysis*.

| step | variable entered | partial $r^2$ | F | p>F | Wilk's lambda | $R^2$ |
|---|---|---|---|---|---|---|
| 1 | K | 0.2469 | 32.789 | 0.0001 | 0.7531 | 0.2469 |
| 2 | Ni | 0.0710 | 7.567 | 0.0071 | 0.6996 | 0.3004 |
| 3 | Fe/Mn | 0.1123 | 12.398 | 0.0007 | 0.6210 | 0.3790 |
| 4 | Mg | 0.0440 | 4.462 | 0.0372 | 0.5937 | 0.4063 |
| 5 | Mn | 0.0317 | 3.142 | 0.0795 | 0.5749 | 0.4251 |
| 6 | ADH | 0.0360 | 3.550 | 0.0626 | 0.5542 | 0.4458 |

* statistics given at each step are: $r^2$, the squared partial correlation; F, prob > F based on one-way analysis of covariance; Wilk's lambda (the associated F approximation prob > F [not shown] was 0.000 for each step); $R^2$, the average squared canonical correlation (prob > $R^2$ [not shown] was 0.000 for each step).

Table 4. Within-group covariance matrices*.

| | | mesic | | | | | |
|---|---|---|---|---|---|---|---|
| variable | | K | Fe/Mn | Ni | Mg | Mn | ADH |
| | K | 1.0 | -0.0930 | 0.1907 | 0.3405 | 0.0403 | -0.0076 |
| | Fe/Mn | -0.0025 | 1.0 | 0.0149 | -0.0445 | -0.0113 | -0.0186 |
| w | Ni | -0.0239 | -0.4833 | 1.0 | 0.0562 | 0.0299 | 0.0311 |
| e | | | | | | | |
| t | Mg | 0.4017 | 0.1082 | -0.2421 | 1.0 | 0.0184 | -0.0586 |
| | Mn | -0.3788 | -0.5312 | 0.4605 | -0.4584 | 1.0 | 0.0120 |
| | ADH | 0.1311 | -0.1513 | -0.0570 | 0.1135 | -0.2009 | 1.0 |

* the mesic group had 26 degrees of freedom, the wet had 74.

As this value was significant at the 0.10 level, the within covariance matrices (Table 4) were used in the discriminant function rather than the pooled covariance matrix (Kendall and Stuart, 1968). The means and standard deviations of the root coating and ADH data of the seedlings from the two soil wetness classes (as determined by cluster analysis) are presented in Table 5.

Under most situations in which one would want to utilize root response variables to identify site-wetness categories, a prior classification would not be available. Therefore, the prior probability of membership into either group was considered equal, and the procedure assigned profiles on the basis of the following quadratic discriminant function:

$$D^2_J(X) = (X-\bar{X}_J)' \, COV_J^{-1} \, (X-\bar{X}_J) + \ln |COV_J|$$

where J is the subscript referring to wetness class, and X and $\bar{X}$ are the mean and individual root variable matrices, respectively (SAS, 1982). The natural log of the determinant of the covariance matrices was -0.523 for the wet group and -12.240 for the mesic group. This formula was applied to the vector of predictor variables for the observation in question, using the appropriate mean vector and covariance matrix for both wetness classes in turn. The observation was assigned to the group for which its generalized distance, $D^2(X)$, was smaller.

Table 5. Means * and standard deviations of ADH and all measured root coating constituets by soil wetness category.

| | mesic class (n=27) | | wet class (n=75) | |
|---|---|---|---|---|
| | mean | s | mean | s |
| ADH | 45.87 | 29.775 | 59.67 | 97.182 |
| Al | 28.36 | 21.494 | 72.27 | 67.651 |
| As | 15.43 | 15.946 | 34.73 | 29.923 |
| Ca | 616.00 | 159.293 | 569.81 | 382.393 |
| Fe | 834.61 | 970.197 | 5632.94 | 5056.766 |
| Fe/Mn | 13.14 | 16.998 | 56.42 | 76.636 |
| K | 3543.80 | 1387.661 | 1963.60 | 1168.967 |
| Mg | 207.14 | 73.567 | 185.90 | 157.683 |
| Mn | 82.78 | 43.175 | 315.93 | 374.681 |
| Ni | 1.27 | 1.284 | 2.27 | 1.584 |
| P | 222.51 | 116.152 | 309.96 | 327.158 |
| Zn | 5.59 | 3.088 | 10.36 | 20.973 |

* units of ADH (alcohol dehydrogenase) are in umoles/g fr. wt/h; units of root coating constituents are in ug/g fr. wt, except Fe/Mn which is the ratio of Fe to Mn calculated on a per plant basis.

When the above formula was applied to all observations in the data set, the a posteriori probability of correct classification of the wet plots was 96.0% and that of the mesic plots was 96.3% However, this estimate of accuracy is known to be biased in that it tends to underestimate the actual probability of misclassification (Green, 1978 p. 176; Lachenbruch and Mickey, 1968). Therefore, the probability of correct classification was determined using the jackknife technique, which is unbiased (Lachenbruch and Mickey, 1968). Each observation was assigned to a wetness class using the distance model derived for the data set exclusive of that observation (Sokal and Rohlf, 1981). This method indicated that the overall probability of correct classification of the wet plots was 92.0%, and 88.9% for the mesic class (Table 6).

SEM Examination:

Root samples from both plot one and three of the Spring Bayou transect showed some degree of coating and the presence of fungal hyphae (Fig. 3, a&b). The sample from plot three (Fig. 3, a) was amorphous, and according to Chen et al. (1980), the conspicuous crack is characteristic of iron oxyhydroxide (FeOOH). In contrast, the coating on the sample from plot one was thinner and exposed epidermal cells were visible in places (Fig.3, b). Root hairs and hyphae projected through the root coating of both samples.

Table 6. Summary of Jackknife determinations of correct classifications of site wetness category based on predictor variables: K, Fe/Mn, Ni, Mg, Mn and ADH.

| | Pearl River | Red River | Spring Bayou | Quimby | Over-All |
|---|---|---|---|---|---|
| number of root samples from: | | | | | |
| mesic plots | 0 | 12 | 0 | 15 | 24 |
| wet plots | 29 | 6 | 24 | 16 | 75 |
| percent correctly grouped: | | | | | |
| grouped into: | | | | | |
| mesic | - | 91.7 | - | 86.7 | 88.9 |
| wet | 100.0 | 100.0 | 91.7 | 75.0 | 92.0 |

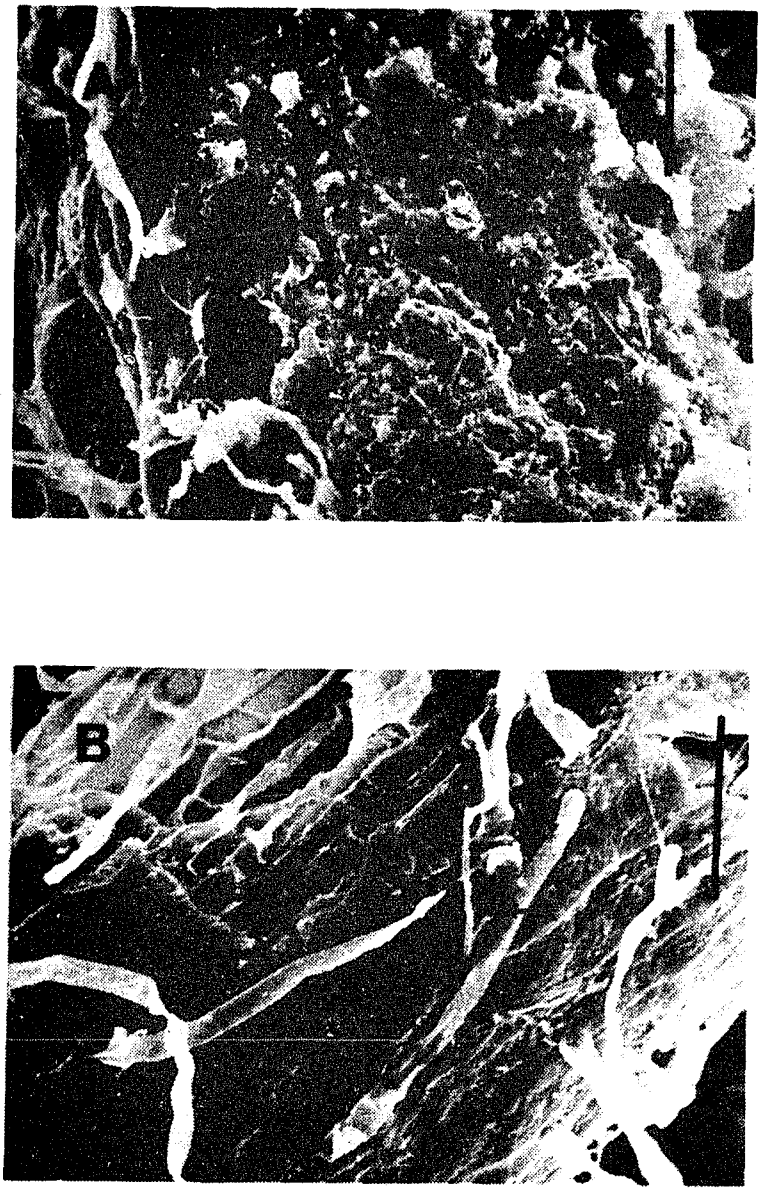


Figure 3. Scanning electron micrographs of green ash roots from Spring Bayou transect at plots three (a) and one (b). Root coatings, root hairs, and mycorrhizal hyphae can be seen in both samples. The large crack in the upper-center of (a) is characteristic of Fe-OOH coatings (Chen et al., 1980).

# DISCUSSION

The material that collects at the root-soil interface is not a part of the root, yet it is distinctly different and isolable from the surrounding soil. From an ecological point of view, root coatings offer a unique vantage point in the analysis of plant-soil interactions. Under waterlogged soil conditions, oxidation-reduction chemistry will play a dominant role in the formation of coatings around oxidizing roots and their composition should be an integrated (over time) function of soil X rhizosphere chemistry. Therefore, some constituents of these coatings (and ADH, which is known to be stimulated under anaerobic conditions) should be useful in differentiating wet from mesic sites. The results of this experiment substantiated this hypothesis: the root coatings and anaerobic respiration were distinctly different enough on wet and mesic sites to be useful in the development of a model for site wetness classification.

## General Mechanisms of Root Coating Formation:

The mechanisms governing deposition of materials on root surfaces in soil is an extremely complex and relatively unexplored area. The oxidized zone around roots in reducing environments is in some ways analogous to the thin oxidized surface layer which is often characteristic of submerged soils and sediments (Gambrell and Patrick, 1978). However, there are some differences that should be taken into consideration: e.g. rhizosphere pH and mycorrhyzial

associations.

Mycorrhizae can enhance root uptake of many nutrients, including: P, K, Ca, Mg, Fe, and Zn, through mycelial penetration of the soil, effective nutrient absorption and storage, and increased root volume and longevity (Bowen, 1973). Certain mycorrhizal fungi will tolerate waterlogged soil conditons, although most do not (Anderson et al., 1984). It has been demonstrated under laboratory conditions (Read and Armstrong, 1972) that the growth of these symbionts under anaerobic conditions is dependant upon the diffusion of oxygen from the roots of the host plant. Green ash is able to oxidize its rhizosphere, and evidence of mycorrhizal relationships were observed in samples from wet plots (Fig. 3, a&b). There is therefore no reason to suspect that mycorhyzae exerted appreciable differences in root coatings taken from mesic versus wet plots.

Roots are known to induce pH changes within their rhizospheres (Bowen, 1973; Godo and Reisenauer, 1980). The pH increases if root uptake of anions exceeds cation uptake, and vice versa (Riley and Barber, 1971). This can influence nutrient solubility; for example, root exudates such as hydroxy-carbonates have been shown to increase Mn availability (Godo and Reisenauer, 1980). However, green ash rhizosphere pH was not measured.

The variables chosen by the stepwise discriminant function were those which most effectively separated the group centroids of the wet and mesic plots. This choice depended on mean separation and variance of each variable as compared across the two groups. Had a different grouping of plots been developed, then a diffent set of

variables would have likely been selected as predictors. The overall differences between each of the root variables from the wet and mesic samples were in agreement with certain well-documented processes known to affect their chemical dynamics. Since these variables can be divided into groups of elements which share similar chemical behaviour in these systems, a brief discussion of the possible underlying mechanisms is warranted. As the data is only correlative, only a general, hypothetical treatment of these observations is possible at the present time.

PH and Cation Exchange Reactions: K, Mg, and Ca:

Of the elements analysed, K was the best predictor variable, and decreased with increased site wetness. Calculations, based on root interception and root uptake, and the use of autoradiographs have shown that $^{86}Rb$, an analog of K, (Walker and Barber, 1962), and Ca (Barber and Ozanne, 1970), and Mg (Al Abbas and Barber, 1964; Oliver and Barber, 1966) can accumulate at the root-soil interface when the supply to the root by mass flow or diffusion exceeds the uptake by the root (see Barber 1984, ch 4). This would seem to be a likely explanation of the relatively high levels of K and Ca on the root surfaces of the mesic class samples. The decline in K, Ca, and Mg, on the roots from the wet plots, may be "a secondary effect of submergence and reduction, chiefly solvent action of $CO_2$ and cation-exchange reactions (Ponnamperuma p.321, 1964)."

Oxidation-Reduction Reactions: Fe, Mn, Fe/Mn:

The reduced forms of iron and manganese are soluble, while their oxidixed forms are not (see Gambrell and Patrick, 1978). Many flood tolerant species (e.g. Armstrong, 1968; Hook et al, 1970; Keeley, 1979), including Fraxinus pennsylvanica (Sena Gomes and Kozlowski, 1980), are able to oxidize their rhizospheres under reducing soil conditions. This process can result in a plaque or coating of oxidized iron, manganese, and co-precipitated materials (Chapter Six). The precipitation of reduced iron onto oxidizing root surfaces has been well documented in many flood-tolerant species (Bartlett, 1961; Bacha and Hossner, 1977; Green and Etherington, 1977; Chen et al., 1980; Mendelssohn and Postek, 1982; Taylor et al., 1984). Although their variation (Table 5) diminished their effectiveness as predictor variables in this experiment, the increase in the mean levels of Fe and Mn on the wet plots was striking (Table 5). Iron increased by a factor of seven, and Mn by four. Thus, although variable, the oxidation-reduction reactions of Fe and Mn were obviously of primary importance to the overall chemistry of root plaque formation.

It has been suggested (see Chapter Six) that the Fe/Mn ratio should be a sensitive indicator of root oxidation in reduced soils because: 1) Mn oxidation is much slower than iron, 2) the critical redox potential for iron oxidation is lower than that of Mn oxidation, 3) these soils generally contain greater amounts of iron than manganese , and 4) the ratio cancels differences in absolute amount between observations. The Fe/Mn values and Mn were much

higher on roots from the wet plots (Table 5), and were selected as predictor variables (Table 3). Iron was likewise much higher among the wet root coating samples, but was not among the predictor variables presumably because because of its high standard deviation (Table 5).

Sorption Reactions: Ni, P, Al, As, and Zn:

The dynamics of Ni, P, Al, As, and Zn, are indirectly influenced by changes in the soil redox potential: their solubility is not governed by valency changes as is the case with Fe and Mn. When reduced Fe and Mn are oxidized they form oxides and oxyhydroxides (Bacha and Hossner, 1977; Sims and Patrick, 1978; Chen et al., 1980). The accumulation of the above elements on roots from the wet plots is probably related to the oxidation of Fe and Mn at the root surface, and the concomitant occlusion of various complexes. Aluminium- and Fe-phosphates are only slightly soluble (Patrick et al., 1973) and can be occluded during the formaton of hydrated iron and aluminum oxides (Mahapatra and Patrick, 1969; Khalid et al., 1977). It has been shown that Fe and Al interact synergistically in phosphorous sorption into iron-phosphorous and aluminum-iron-phosphorous complexes (Pritchard et al., 1984). The hydrous oxides of Al, and those of Fe and Mn, act as absorbants for phosphate and some other elements, including: Ni (Bowen p. 54, 1979), As (Bowen p.53, 1979), and Zn (Sims and Patrick, 1978); which were all greater in the root coatings of the wet plots (Table 5).

ADH:

Numerous studies have demonstrated the stimulation of alcoholic fermentation in flood-tolerant species under anoxic conditions (e.g. John and Greenway, 1976; Smith and Ap Rees, 1979; Rumpho and Kennedy, 1981; Tripepi and Mitchell, 1984). This response has also been demonstrated in green ash under short term (Hook and Brown, 1973) and relatively long term (Chapter Six) experiments. These results have considered this to be primarily a result of insufficient oxygen for aerobic respiration. Root ADH in green ash can also be stimulated by high levels of $CO_2$ in the root environment (Chapter Five), a situation of common occurrence in flooded soils (Ponnamperuma et al., 1966; Hook et al., 1970).

Mean ADH was greatest from the wet plot samples (Table 5), and was included in the discriminant function; however, based on its low $r^2$ (Table 3), its contribution to the predictive capacity of the model was not great. The standard deviation of this variable was high (Table 5), especially among roots from the wet areas. There are several possible sources of variation in ADH activity. Other studies (Keeley, 1979; Pedrazzini and McKee, 1984) have shown that ADH activity can decline after prolonged exposure to waterlogged soil conditions to control levels. This response probably results from the increased aeration of roots formed under anaerobic conditions (Keeley, 1979; Pedrazzini and McKee, 1984). Flooding intensity can also affect ADH activity. The ADH activity of Spartina alterniflora roots growing in a controlled soil suspensions was inversely related to redox potential, a measurement of anaerobic intensity (DeLaune et

al., 1984). Thus, differences in the length of time of exposure to flooded conditions and the intensity of anaerobiosis could have accounted for some of the observed variation in ADH activity.

The Model:

The root variables selected by the stepwise discriminant function (Table 3) proved to be effective predictors of site-wetness. The effectiveness of the discriminant function (Table 6) did not appear to correspond to differences in the pH or percent base saturation in the A horizon of the various transects (Table 1), two parameters which could have influenced root coating formation. The accuracy of determination was poorest at the Quimby transect (Table 6). Most of the errors were from the samples taken at plot three (wet): three of the eight samples were classed as mesic. This plot had quite a lot of microtopographic variation, and could perhaps be classified as partly mesic and partly wet.

The overall accuracy of site-wetness classification can likely be improved above the levels presented here. The variation in root coating would have been less had the determinations of root coating constituents been based on a dry-weight rather than fresh-weight basis. Also, the field and laboratory work involved in the ADH sampling and assay is inordinate in view of the amount of information gained (Table 3). Sampling time could have been used more effectively to collect additional root coating samples.

Green ash has several characteristics which make it a good species for the collection of the type of data used in this discriminant

function. Rhizosphere oxidation under waterlogged soil conditions appears to be an essential criterion. This enhances the seedlings' chances of survival at the wetter end of the soil moisture gradient (see Chapter Six), and favors the formation of root coatings characteristic of a reduced soil environment. It should be mentioned that an identical experiment with water oak (Quercus nigra L.) failed. This species apparently does not effectively oxidize its rhizosphere and its root coating composition does not respond to waterlogging as dramatically as that of green ash (Chapter Six). The high mortality rate of oak at the wetter plots precluded an adequate sample from any transect (data not shown). The suitability of green ash for this type of study was suspected because of its flood-tolerance (Broadfoot and Williston, 1973; Hook and Brown, 1973; Hook, 1984), and its natural occurrance as understory and overstory constituents on mesic and wet plots (Table 1).

## CONCLUSIONS

This study is apparently the first to investigate the potential application of using root coating characteristics and ADH activity to indentify waterlogged sites. The method worked well for most of the sites studied. Further refinement will permit more precise predictive capability. The main advantage of the method is that the field work and sample preparation for the root coating analyses are relatively easy and inexpensive. However, the collection and

analysis of ADH samples is somewhat less convenient, but in this study the root coating data alone would have probably served as an adequate set of predictor variables. The main disadvantage is that the method is dependant upon a fairly flood-tolerant species that oxidizes its rhizosphere under reduced soil conditions and is known to survive in each of the types of sites one wishes to classify, e.g., in a subcanopy situation similar to this study, some tolerance to understory conditions is a prerequisite. However, it is likely that many species, endemic or transplanted, woody or non-woody, would make good candidates. The possibility of using mature, endemic individuals should be addressed because they could provide an abundant source of readily available information. The approach presented in his paper merits further study, because it could provide an efficient indicator of BLH "wetlands", and shed light on plant-soil interactions under flooded soil conditions.

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# VITA

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**DOCTORAL EXAMINATION AND DISSERTATION REPORT**

**Candidate:** Billy J. Good

**Major Field:** Marine Sciences

**Title of Dissertation:** Effect of Anaerobic Root Environments on *Fraxinus pennsylvanica* Marsh. and *Quercus nigra* L.

**Approved:**

Major Professor and Chairman

Dean of the Graduate School

**EXAMINING COMMITTEE:**

**Date of Examination:**

September 4, 1985